# EVALUATION OF DIFFERENT GENETIC INTERVENTION APPROACHES FOR THE ENHANCEMENT OF CAROTENOIDS; ASSESSMENT OF ABA AND ANTIOXIDANTS MEDIATED EXTENDED SHELF LIFE IN *SOLANUM LYCOPERSICUM*

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## Declaration of Authorship

I hereby declare that the work presented in this thesis is the original work of the author unless otherwise stated. Original material used in the creation of this thesis has not been previously submitted either in part or whole for a degree of any description from any institution.

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20th August 2016

## Abstract

Carotenoids are important groups of natural pigments belonging to the class of natural products known as isoprenoids. Lycopene is an important carotenoid commonly found in the human diet and represents 50% of carotenoids found in human serum and has a high antioxidant effect in the body. Ketocarotenoids are oxygenated carotenoids also known as xanthophylls, but this specific class of xanthophylls are rarely found in higher plants but are used commercially in animal feedstock especially aquaculture for salmon and shrimp and therefore have a large market value. In this study, different strategies were evaluated for the enhancement and production of high-value carotenoids and ketocarotenoids in tomato fruit. The mechanisms underlying the metabolic phenotype and associated physiological changes were investigated.

Identification and validation of putative transcription factors (TF) followed in three parts of first objective:

1- *APRR2-like* (Arabidopsis pseudo-response regulator-2-like) on chromosome 6. Primary identification on *APRR2-like* homologue gene was initiated which needs more investigation.
2- *ESB9* TF designated as Solyc06g053960. *ESB9* F1 characterisation demonstrate the effect of this gene on decrease of β-carotene level in all of the lines; the next generation characterisation could lead to identification of an effective TF on pigmentation
3- putative candidate genes/regions in the *S.pennelli* sub ILs of 2-4 and 2-5 (Q1968 line) were studied as one strategy. The Q1968 line results indicate a significant increase in lycopene and some other metabolites. Firstly a pattern of effect was proposed for responsible regulators based on stages of ripening. Secondly two probable types of pattern that the introgressed region genes can operate for their effects were investigated.

In another approach nonendogenous ketocarotenoids in tomato have been increased by combining biosynthetic optimisation [astaxanthin biosynthesis in CrtZW line (β-carotene ketolase/hydroxylase)] and transcriptional regulators [DET1(De-etiolated 1) and APRR2].

In CrtZW.APRR2 line no dramatic increase was found. Further investigation to elucidate the biochemical molecular mechanisms underlying the CrtZW.APRR2 phenotype provide a deep insight to choosing the best effective candidates for

improving high-value compounds based on the TF mechanism which affect pigmentation and ripening.

Through genetic crossing a new variety termed CrtZW.DET1 exhibited a significant increase in carotenoids both carotenes such as lycopene and high value ketocarotenoids. These genotypes also displayed delayed ripening the hypothesis proposed involved the modulation of abscisic acid (ABA) content in the fruit due to the depletion of β ring derived carotenoids. Associated changes in cell-wall degradation enzyme activities, hormones level and cell wall compound measurements were also observed. These data have revealed a new mechanism for improving the shelf life characteristics of fleshy fruits via introducing new pathways to plant.

Firmness and weight alterations trend in shelf life experiments of CrtZW.DET1 line demonstrate a value added to this line for its unique shelf life property and its potential for improving the other crops quality.

*In the Name of GOD, the Compassionate the Merciful*


## Acknowledgement

Nothing happens in the universe unless *Allah* wants it.

I want to dedicate this thesis to my father Mohsen Moghadaszadeh who I have done my Ph.D. for him. My father's spiritual and financial supports provide this opportunity for me. And also I want to dedicate it to Mahdi Shafaei, my dear husband.

I would like to say my deep gratitude to Mahdi for all of his love, supports and helps to overcome all of the abroad living difficulties and doing the Ph.D.; without him I could not complete my study. I like to thanks my mother Maryam Esteghamatian, my brother Mohammad and my sister Zahra for their enthusiastic encouragement during my study.

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At the end I would like to bring a quote from one of the greatest human in the world Imam Sadeq (peace be upon him):

**Real science is the light which *Allah* puts in the heart of who wants.**

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## List of abbreviations

| | |
|---|---|
| Abs | Absorbance |
| AC | Ailsa craig |
| APRR2 | Arabidopsis pseudo-response regulator-2 |
| ATP | Adenosine-5'-triphosphate |
| BSA | Bovine serum albumin |
| Br | Breaker stage |
| C | Celsius |
| 35S | Cauliflower mosaic virus 35S promoter |
| CCD | Carotenoid cleavage dioxygenase |
| Chl | Chlorophyll |
| CoA | Coenzyme A |
| CRTB | Bacterial phytoene synthase |
| CRTE | Bacterial GGPP synthase |
| CRTI | Bacterial phytoene desaturase |
| CRTISO | Carotenoid isomerase |
| CRTW | β-carotene ketolase |
| CRTZ | β-carotene hydroxylase |
| Ct | Cycle threshold |
| Cv | Cultivar |
| CYC-B | Fruit specific lycopene β-cyclase |
| DDB1 | Damaged DNA binding protein 1 |
| DET1 | De-etiolated 1 |
| $dH_2O$ | Distilled water |
| DMAPP | Dimethylallyl diphosphate |
| DNA | Deoxyribonucleic acid |
| dsDNA | Double stranded DNA |
| DW | Dry weight |
| DXP | 1-Deoxy-D-xylulose 5-phosphate |
| DXS | 1-Deoxy-d-xylulose 5-phosphate synthase |
| DXR | 1-Deoxy-D-xylulose 5-phosphate reductoisomerase |
| EDTA | Ethylenediaminetetraacetic acid |
| F | Fraction |
| FAD | Flavin adenine dinucleotide |
| FAOSTAT | Food and Agriculture Organisation of the United Nations |
| FDA | Food and Drug Administration |
| FPP | Farnesyl diphosphate |
| GA3P | Glyceraldehyde-3-phosphate |
| GA | Gibberellic acid |
| GC-MS | Gas Chromatography Mass Spectrometry |
| GGPP | Geranyl geranyl diphosphate |
| GGPPS | Geranyl geranyl diphosphate synthase |
| GM | Genetically modified |
| GMO | Genetically modified organism |
| h | Hour |
| Hp-1 | High pigment 1 |
| Hp-2 | High pigment 2 |
| Hp-3 | High pigment 3 |
| HPLC | High performance liquid chromatography |
| IPP | Isopentenyl diphosphate |
| LB | Luria Broth medium |

| | |
|---|---|
| LCYE | Lycopene ε-cyclase |
| LCYB | Lycopene β-cyclase |
| LC-MS | Liquid chromatography mass spectrometry |
| Min | Minute |
| MG | Mature green stage |
| MVA | Mevalonate pathway |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate Hydrogen |
| NCED | 9-Cis-epoxycarotenoid dioxygenase |
| NXS | Neoxanthin synthase |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PDA | Photo-diode array |
| PDS | Phytoene desaturase |
| PG | Plastoglobule |
| PIF | Phytochrome-interacting factor family |
| PIF5 | Phytochrome-interacting factor 5 |
| PSI | Photosystem I |
| PSII | Photosystem II |
| PSY-1 | Fruit specific phytoene synthase 1 |
| PSY-2 | Phytoene synthase 2 |
| qPCR | Quantitative PCR |
| QTL | Quantitative trait loci |
| RI | Retention index |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcription PCR |
| RTqPCR | Quantitative real time reverse transcription |
| SD | Standard deviation |
| Sec | Second |
| SIERF6 | Ethylene response factor |
| TILLING | Targeting Induced Local Lesions IN Genomes |
| Tris | Tris-hydroxymethyl-aminomethane |
| UPLC | Ultra High Performance Liquid Chromatography |
| VDE-1 | Violaxanthin de-epoxidase |
| ZEP-1 | Zeaxanthin epoxidase |
| Z-ISO | 15-Cis- ζ-carotene isomerase |

# CHAPTER 1: INTRODUCTION

## 1.1 Tomato fruit: An economically important crop, high nutritional value and a model of fleshy fruits in plant science.

On the basis of volume consumed, tomato is one of the most important crops in the world. Tomato (*Solanum lycopersicum*) is the second most important vegetable crop with total world production of 163.4 million tons worth $59.9 billion (FAOSTAT Database, 2013). Short crop duration, high yield, capability of tomato production through whole year in poly tunnels, fields or glasshouses and its high nutrition value are important economic criteria, which result in increasing cultivation of this crop. In the last decade the production increased over 35 million tones worldwide based on FAOSTAT data from 2004 (Figure 1-1). Tomato is used widely in different forms of raw and processed food such as ketchup, soup, paste, purée and sauce. Nutritionally tomatoes are rich in vitamins (such as vitamin A) and organic acids, minerals and dietary fibres (USAD, 2016). Table 1-1 represent detailed tomato nutritional facts.

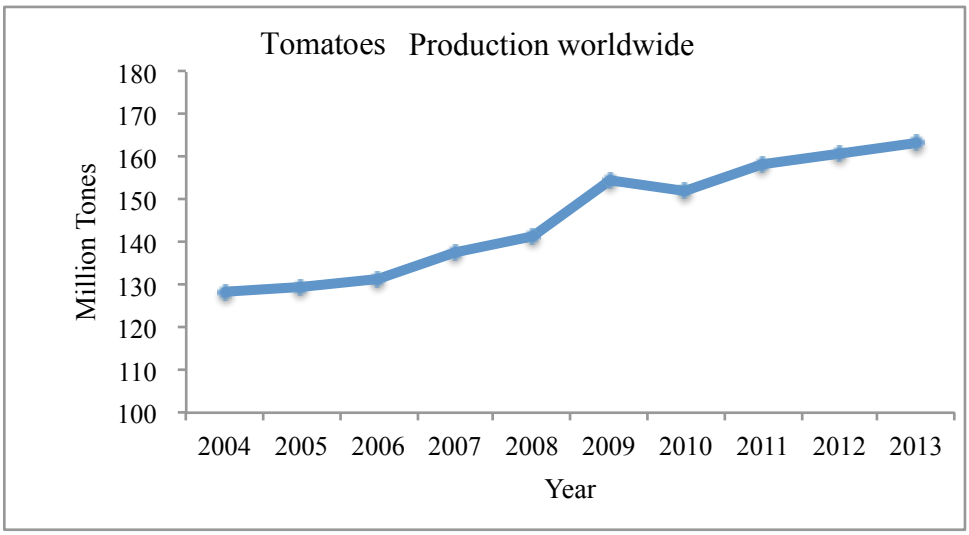



Figure 1-1 The trend of growth in tomato production from 2004 to 2013 worldwide (FAOSTAT, 2016 http://faostat.fao.org/)

Many biochemical and molecular studies have been done on tomato and translated into biotechnological applications. Tomato is a model for fleshy fruits, predominantly because of its short generation and simple diploid genetics and in addition rich omics data resources that are now available (Giovannoni, 2001). The ripening phenotype is very easy for detection, large germplasm resources are available and many valuable mutants are available such as inhibited or altered ripening mutants (Meissner et al., 1997; Tanksley et al., 1992). Tomato was the first crop in fleshy fruits which

underwent whole genome sequencing and there are a large platforms of tools for computational analysis (Barry and Giovannoni, 2007; Mueller et al., 2005).

Table 1-1 Nutrition facts of red ripe raw tomatoes. Data compiled from USDA National Nutrient Database for Standard Reference (USAD, 2016)

Kcal represent kilo calorie, g: gram, mg: milligram, µg: microgram and IU: international unit

| **Nutrient** | Unit | 1Value per 100 g |
|---|---|---|
| Water | g | 94.52 |
| Energy | kcal | 18 |
| Protein | g | 0.88 |
| Total lipid (fat) | g | 0.2 |
| Carbohydrate, by difference | g | 3.89 |
| Fiber, total dietary | g | 1.2 |
| Sugars, total | g | 2.63 |
| **Minerals** | | |
| Calcium, Ca | mg | 10 |
| Iron, Fe | mg | 0.27 |
| Magnesium, Mg | mg | 11 |
| Phosphorus, P | mg | 24 |
| Potassium, K | mg | 237 |
| Sodium, Na | mg | 5 |
| Zinc, Zn | mg | 0.17 |
| **Vitamins** | | |
| Vitamin C, total ascorbic acid | mg | 13.7 |
| Thiamine | mg | 0.037 |
| Riboflavin | mg | 0.019 |
| Niacin | mg | 0.594 |
| Vitamin B6 | mg | 0.08 |
| Folate, DFE | µg | 15 |
| Vitamin B12 | µg | 0 |
| Vitamin A, RAE | µg | 42 |
| Vitamin A, IU | IU | 833 |
| Vitamin E (alpha-tocopherol) | mg | 0.54 |
| Vitamin D (D2 + D3) | µg | 0 |

| | | |
|---|---|---|
| Vitamin D | IU | 0 |
| Vitamin K (phylloquinone) | µg | 7.9 |
| **Lipids** | | |
| Fatty acids, total saturated | g | 0.028 |
| Fatty acids, total monounsaturated | g | 0.031 |
| Fatty acids, total polyunsaturated | g | 0.083 |
| Fatty acids, total trans | g | 0 |
| Cholesterol | mg | 0 |

## 1.2 Tomato diversity

Initially tomato was named as pomi d'oro and mala aurea (golden apple) also known as poma amoris (love apple). The recent name "tomato" comes from Nahua Tongue of Mexico where they used tomato fruit as "tomatl" (Goodenough, 1990). Tomato domestication is relatively recent within the last 400 years. It is believed that the first domestication happened in maze fields by ancient Mexicans in Peru. Therefore, Peru is considered as the first centre of diversity for the genus. Cultivated tomatoes originated from Andean region in South America introduced to south and west part of Europe continent after the discovery of America (Davies and Hobson, 1981). Arcanum, Neolycopersicon, Eriopersicon and Eulycopersicon are main 4 subgenera of *Solanum* which represent different varieties of tomato plants. Table 1-2 represents four groups of the *Solanum* species (Tomatoes). In the past 20 years tomato germplasm was utilized using wild species as sources of genetic variation for crop improvement; for instance, as sources of variation for disease/insect resistances, fruit quality and abiotic stress tolerances. As a result many valuable introgressed lines from wild type varieties with specific traits have been generated and consequently an enhancement in molecular genetic variation of the regions that were introgressed from wild type species (Peralta and Spooner, 2006).

*Solanum pennellii* is a wild tomato species originating from the Andean regions in South America (see Table 1-2). Due to its extreme stress tolerance and different morphology such as green fruits, this variety studied as an important donor of germplasm for *Solanum lycopersicum* the cultivated tomato. Introgression lines (ILs) which are genomic regions of *S. lycopersicum* that are replaced with the corresponded parts of *S. pennellii* were used to create numerous quantitative trait loci (QTLs) for

pigments, plant morphology, taste and aroma, abiotic stress tolerance and heterosis (Eshed and Zamir, 1995; Lippman et al., 2007; Liu et al., 2003; Bolger et al., 2014).

Table 1-2 Characteristics and origins of cultivated tomato and wild species (Adapted from Jasionowicz, 2012; Maxted et al., 2014; Peralta et al., 2005; Taylor, 1986; Goodenough, 1990).

| Subgenera | Species | Characteristics | Origin |
|---|---|---|---|
| Eulycopersicon group | *S. lycopersicum* (cultivated tomato) | Self-pollinated | Mexico, central and southern America |
| | *S. lycopersicum* var. cerasiforme (cherry tomato) | Resistance to fungus and root rotting | Central and southern America, the region of Andean foothills |
| | *S. pimpinellifolium* (currant tomato) | Resistance against diseases, improved composition and colour | Ecuador and north part of inter-Andean regions in Peru |
| | *S. cheesmaniae* | Fruit stalks are jointless, tolerance for salt | Galapagos islands |
| Eriopersicon group | *S. peruvianum* | Resistance to disease and pests, vitamin C content | High Andes in Peru and some coastal regions |
| | *S. habrochaites* | Resistance to disease and pests, tolerance for low temperature, improved fruit colour | Peru and Ecuador, high altitudes |
| | *S. chilense* | Cold, virus drought and disease resistance | Northern Chile and southern part of Peru |
| | *S. chmielewskii* | Improved composition, fruit colour and total solids | Peruvian Andes, high region |
| | *S. parviflorum* | Improved composition, fruit colour and total solids | Peruvian Andes, high region |
| Arcanum group | *S. arcanum* | An extremely variable species, consisting of four weakly defined morphotypes | Inland Andean valleys in northern Peru |
| | *S. chmielewskii* | Flowers larger, green fruit | Upper Apurimac valley of Peru and Sorata valley Bolivia |
| | *S. neorickii* | Small fruits and flowers, green fruits | Ecuador to southern Peru |
| Neolycopersicon group | *S. pennellii* | Stress tolerance and green fruits | Andean regions in South America |

## 1.3 Morphology of the tomato fruit

Tomatoes are diploid plants similar to most of *Solanaceae* species. Normally the chromosome numbers are 12. This easy to grow species is grown throughout the year specially the commercial varieties using poly tunnels and green housed in winter. The tomato flower and fruit morphology are represented in Figure 1-2. Most of tomato flowers are self-pollinated and mainly commercial tomato fruits weigh approximately 100 grams. Many tomato varieties grow to maximum 3 meters in height but have a weak stem, which led to some difficulties for carrying the fruit weights (Kimura and Sinha, 2008; Taylor, 1986). There are thousands of tomato varieties and their classification can be difficult, but the last method of classification is based on the fruit shape (Jasionowicz, 2012).

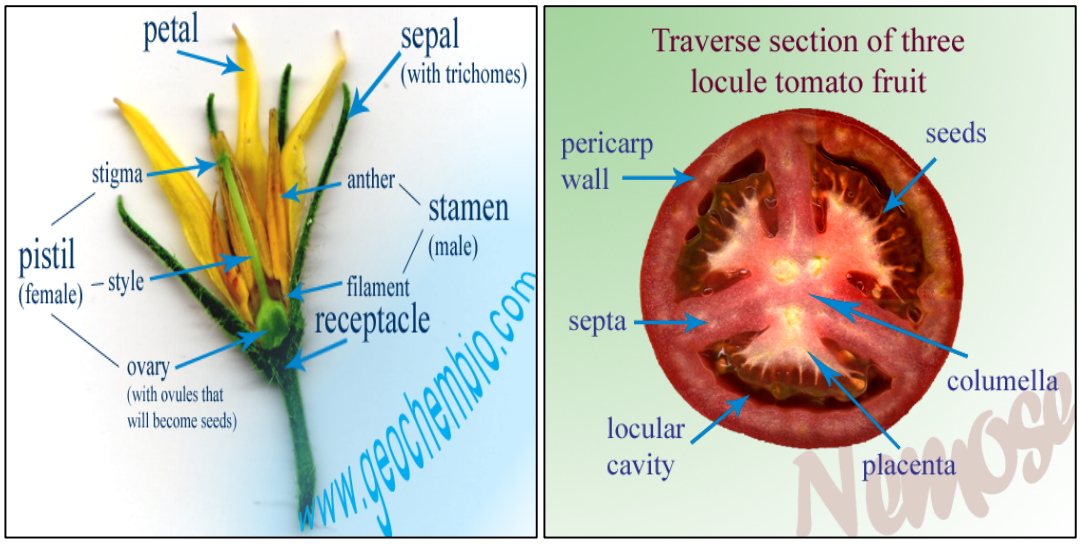



Figure 1-2 Tomato flower and fruit morphology (http://www.geochembio.com/biology/organisms/tomato/)

## 1.4 Fruit development

Fleshy fruits are botanically different to each other in their stages of fruit development. Fruits such as tomato and grape are derived from a single ovary and are in the true berry category of fleshy fruits. Fruit set is the initial stage of fruit development after the fertilization step. Fruit set is followed by cell division and consequently cell expansion. The two cell division and expansion contribute to the fruit growth phase, which attain the maximum size of fruit (Klee and Giovannoni,

2011; Seymour et al., 2013b) (see Figure 1-3). The cellular, hormonal and metabolite alterations within the stages of ripening are explained in sections 1.4.1, 1.4.2 and 1.5.

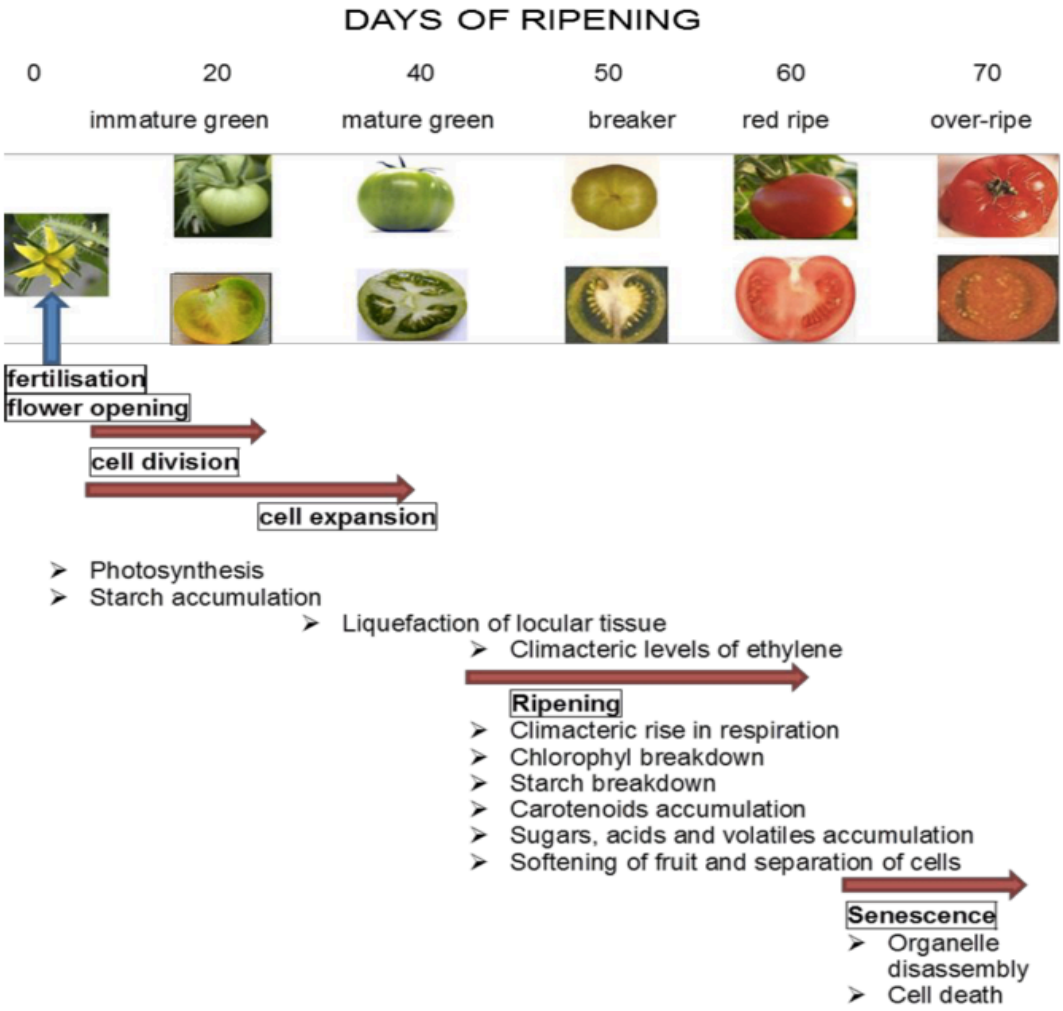



Figure 1-3 Development and ripening of tomato fruit (Jasionowicz, 2012)

The numbers represent days post flower opening; and stages of ripening defined as: immature green, mature green, breaker, red ripe and over-ripe stages. Arrows indicate the cellular, molecular and metabolite alterations within the stages of ripening.

### 1.4.1 Fruit set

Fruit set is the very first stage in fruit development which relies on the successful pollination and fertilization of one pollen nuclei with the egg cell and the other pollen combines with the two haploid polar nuclei to produce a triploid cell (Raghavan, 2003). These events, which result in production of seeds, control cell division and fruit growth in an organized manner. Three hormones of auxin, gibberellins and cytokinin play important roles in regulation of fruit set. The level of these three

hormones elevate at the process of fruit set. The individual treatment of these hormones just partially can induce fruit development and their combined application results in normal fruit development in the absence of fertilization (Crane, 1964; Kumar et al., 2014; Gillaspy et al., 1993; Mariotti et al., 2011). It is reported that the interaction between auxin and gibberellin signalling pathway is an essential factor in fruit set promotion; evidence suggested that auxin, by controlling the gibberellin level, promotes fruit set and growth (Carrari et al., 2006; Dorcey et al., 2009). In addition to auxin and gibberellin, cytokinin also play role in fruit set. The endogenous level of this hormone correlates directly with fruit growth and specially induces cell division (Matsuo et al., 2012; Mariotti et al., 2011). Ethylene and brassinosteroids are also known to play an important role in fruit set. A transient increase of ethylene in the pistil is observed after pollination, which then decreases after fertilization. It is shown that ovule lifespan is an important criterion in fruit set ability. Ovule senescence also delayed in gibberellin-induced fruits. Therefore a model proposed that possibly ethylene modulates the process of ovule senescence, which may happen by gibberellin signalling (Wang et al., 2009a; Carbonell-Bejerano et al., 2011; Kumar et al., 2014). ABA levels represent decrease at fruit set in result of downregulation of some of its biosynthesis genes (Vriezen et al., 2008). Many factors contribute in process of fruit set but their exact action and relations still needs more investigation.

### 1.4.2 Fruit development and maturation

There is strong relation between seed and fruit development. Seeds are rich sources of various hormones for instance auxin, gibberellin and cytokinin. These hormones stimulate the growth of surrounding tissues and affect the fruit size (Ozga et al., 2003; Kumar et al., 2014). The level of auxin and cytokinin is elevated in seed development concurrently with cell division and cell expansion (Devoghalaere et al., 2012; Blumenfelda and Gazit, 1970). The main role of auxin is in the cell expansion in tomato. A decreasing rate of auxin level was detected from seed (in the middle of fruit) to the outer pericarp section in cell expansion phase suggesting the high auxin level in outer layer of placenta result in expansion of its cells and consequently surrounding the seeds and filling the locular cavity (Pattison and Catala, 2012). ABA also play role in cell expansion as ABA deficient mutants produce smaller fruits (Gillaspy et al., 1993; Nitsch et al., 2012).

Fruit maturation prepare fruit for starting the complex process of ripening. Auxin and cytokinin seems to be the effective hormones in this process; auxin reduction observed in some crops, which seems this reduction is needed for ripening to happen. In RIN mutant (ripening-inhibitor) (see section 1.5.2) higher levels of auxin and cytokinin were detected at breaker stage compared to wild type. Basically the level of auxin is higher in seeds compared to its surrounding tissues that suggest the inhibition of auxin production in surrounding cells or stopping its transport to neighbour tissues which result in seed dormancy and also lets the mature fruit undergo ripening (Davey and Van Staden, 1978; Given et al., 1988; Devoghalaere et al., 2012; Zaharah et al., 2012).

## 1.5 Fruit ripening

Fruit ripening is a complex event with various biochemical, hormonal, structural and cellular alterations affected by environmental stimuli, for instance temperature and light (Dumas et al., 2003). When the cellular division and expansion reaches its maximum capacity, the complex process of ripening is about to happen. As a result of ripening the firm and not edible green fruits turn to nutritionally rich, palatable, soft and coloured fruit. Most of secondary high value compounds accumulate in this phase. Fleshy fruits divide to climacteric and non-climacteric fruits. Climacteric fruits represent a considerable increase in respiration and ethylene biosynthesis to start the ripening for instance tomato, apple, avocado and banana. But these two events are not observed in non-climacterics fruits for example grapes, strawberries and citruses (Klee and Giovannoni, 2011; Seymour et al., 2013b). Cells undergo considerable alterations that are described in Figure 1-4; as the process of ripening progressed, the fruit colour changes owing to pigments accumulation (Klee and Giovannoni, 2011). Conversion of complex carbohydrates to sugars, decrease the acidity of fruits with accumulation of sugars, aroma and flavour occurs (Seymour et al., 2013b). The most important alteration is alteration of green chloroplast to red chromoplasts in ripe tomato fruits in result of alterations such as degradation grana and thylakoids, degradation of the inner membrane envelope of chloroplasts and formation of carotenoid-rich membranous sacs, plastoglobules alterations and etc. (see Figure 1-4). Degrading of cell wall components result in fruit softening, these obvious alterations are regulated by multiple genetic and biochemical pathways (Kumar et al., 2014).

Hormonal alteration, transcriptional regulation, metabolite production and cell wall changes will be reviewed in different sections.

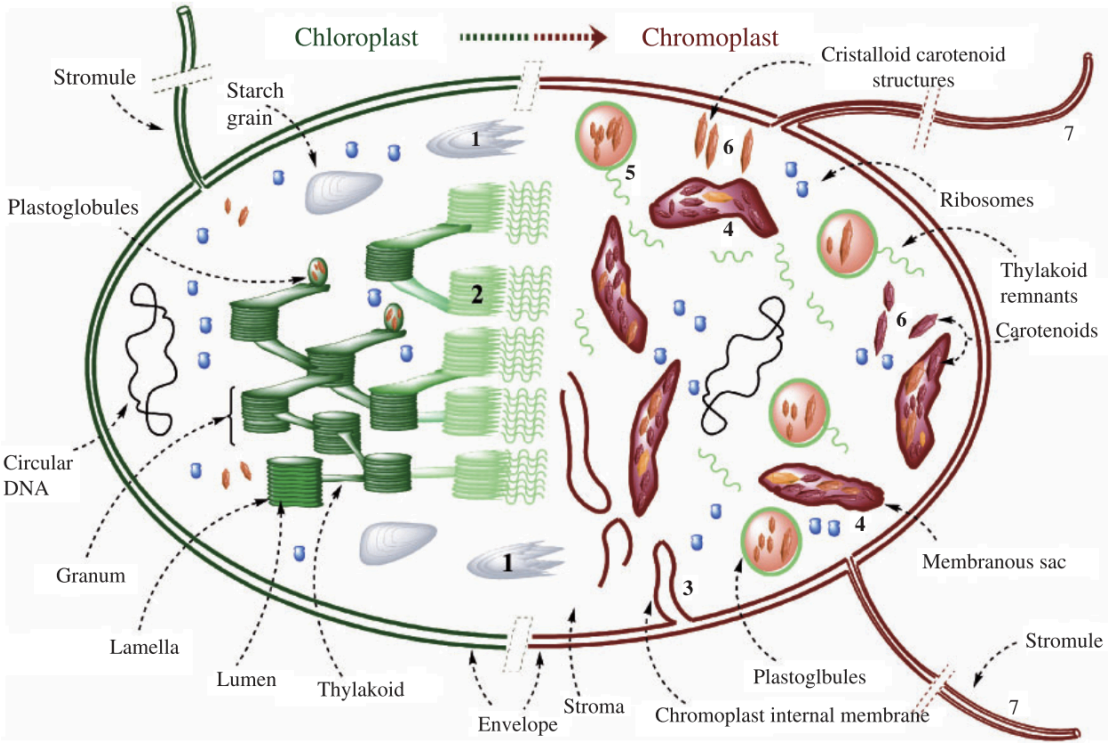



Figure 1-4 Schematic figure of chloroplast to chromoplast transition.

"The scheme indicates the breakdown of starch granules (1) and of grana and also thylakoids (2); the biosynthesis of new membrane form the inner membrane envelope of plastid (3) result in formation of carotenoid-rich membranous sacs (4); elevation in the size and number of plastoglobules (5); appearance of carotenoid-containing crystalloids (6); and the growth in the number of protrusions structures emerging from the plastid envelope, designated stromules (7)" (Egea et al., 2010).

### 1.5.1 Production of high value metabolites

#### 1.5.1.1 Carotenoids and fruit colour

The first alteration in colour is in result of the transformation of chloroplasts into chromoplasts, meanwhile chlorophyll degradation followed by an elevation in β-carotene, responsible for the orange colour at early stages of ripening. Red colour in ripe fruits is caused by high accumulation of lycopene (Rančić et al., 2010). The importance of carotenoids and ketocarotenoids and their functions in nature along with their production pathway will be explained in the next sections.

#### 1.5.1.1.1 Importance of carotenoids and ketocarotenoids

Carotenoids are group of C40 plant pigments. They synthesised in various types of plastids (Hirschberg, 2001). They contain a highly unsaturated polyene chain with alternative single and double bonds, which is a characteristic property of carotenoids (Britton, 1995).

Apocarotenoids are derived from carotenoids by act of oxidative enzymes: carotenoid cleavage oxygenizes (CCOs). They cleave specific double bonds of polyene chain. The various cleaved products can act as hormones, vitamins, chromophores and scent/aroma constituents and signalling compounds. For instance the vitamin A retinoids such as retinol, retinal and retinoic acid (Walter et al., 2010). One of the most important activities of carotenoids in terms of human health is acting as pro-vitamin A. Around 50 carotenoids with β ring end groups, such as β-carotene and zeaxanthin have the capability of Vitamin A production. Specifically β-carotene is the main precursor of retinol or Vitamin A (Fraser and Bramley, 2004; Tang and Russell, 2009). Blindness and growth retardation are linked with Vitamin A deficiency. Also severe diseases including dysentery, autoimmune responses, respiratory and urinary infections are related to Vitamin A deficiency (Tang and Russell, 2009). A rich diet of pro-vitamin A could help in preventing these problems. Apocaroteniods also play role in hormonal functions in plants as well (Giuliano et al., 2003) providing precursors for the synthesis of ABA and other plant hormones (Andersen et al., 2004).

The primary functions of carotenoids in plants consist of different physiological and biochemical roles for example photoprotection, light harvesting and free radical scavenging (Britton, 1995; Demmig-Adams and Adams, 1996). Carotenoids are observed in high quantity in fruit and flower chromoplasts (Cunningham and Gantt, 1998). Carotenoids are responsible for red, yellow and orange colours in many fruits and flowers which act to attract insects for pollination and seed dispersal purposes (Bartley and Scolnik, 1995). In the chloroplasts of plants, algae and cyanobacteria, carotenoids are the integral component of thylakoid membranes (Cunningham and Gantt, 1998). Carotenoids in combination with other proteins, take part in forming the reaction centre complexes and light harvesting centres of photosynthetic apparatus (Howitt and Pogson, 2006). They play role in improving the effectiveness of light capture (Andersen et al., 2004; Obón and Rivera, 2006) via absorbing the wavelengths (blue-green and violet region) that are not absorbable by chlorophyll and consequently transfer the energy to chlorophyll for photosynthesis (Taiz and Zeiger,

2006). They also serve as photo protective agents in the photosynthetic apparatus. The photosynthetic membrane is simply damaged by extra energy that is absorbed by pigments, but did not used in the process of photosynthesis. The excited chlorophyll can react with oxygen molecules and produce singlet oxygen (excited oxygen), which is harmful for cells especially lipid components (Taiz and Zeiger, 2006). Excited carotenoids contain low levels of energy compared to chlorophyll (Bassi et al., 1993), which means they can quench the excited state of chlorophyll rapidly and consequently allow the excess energy to be lost as heat. Non-photochemical quenching (NPQ) which is quenching of chlorophyll fluorescence is another protective and regulatory mechanism. Severe illumination result in excitation of the antenna system; here NPQ acts via adjusting the excitations flow to the PSII reaction centre and quenching excess excitation through conversion to heat therefore protecting the photosynthetic machinery against over-excitation damage (Demmig-Adams and Adams, 1996). Violaxanthin, antherxanthin and zeaxanthin are associated with this process. The increase in the pH gradient across the thylakoid membranes happened in high light and result in conversion of Violaxanthin to antherxanthin and then zeaxanthin (Demmig-Adams and Adams, 2002) this process can be reversed when light levels decrease. Protons and zeaxanthin are attached to the light harvesting antennae proteins result in quenching of excitation by dissipating heat (Demmig-Adams et al., 1996; Horton et al., 1996) confers the importance of xanthophylls in photo protection.

In tomato fruit lycopene is the principal carotenoid responsible for red colour in ripe fruits (Harris and Spurr, 1969). Lycopene is the main membrane-bound antioxidant and serves as highly efficient singlet oxygen quencher in mature fruits (Di Mascio et al., 1989).

Another aspect of carotenoids and ketocarotenoids commercial value, which result in their large markets, is their natural pigmentation properties. They are consumed in industrial colorants especially in the aquaculture and poultry farming industries. Astaxanthin is used for pigmentation of fish and shrimp. Canthaxanthin is using for introduction of a red tone in egg yolk. Carotenoids and ketocarotenoids also are beneficial for human health due to their antioxidant activity (Krinsky, 1989; Rao and Rao, 2007; Ralley et al., 2004).

The antioxidant activity of carotenoids related to their polyene chain (see Figure 1-5 for carotenoid structure) (Britton, 1995). Human diseases, such as heart diseases and

cancers always involve oxidation processes (Gülçin, 2012; Britton, 1995). Antioxidants molecules prevent oxidative damage to cellular components by neutralize reactive oxygen species. Consequently the process of ageing (in result of oxidative damage to cells) and ageing-related diseases decreased and rate of cell death is reduced (Gülçin, 2012; Zhu et al., 2013).

#### 1.5.1.1.2 Carotenoid and ketocarotenoid biosynthetic pathway

##### 1.5.1.1.2.1 Carotenoid biosynthesis pathway in higher plants

There are various genes, which are responsible for control of the carotenoid pathway. Phytoene synthases (PSY) are produced from the expression of two genes *PSY-1* and *PSY-2* responsible for phytoene production. *PSY-1* encodes the fruit specific isoform while the other gene is associated with expression in chloroplast containing tissues such as leaves and mature green fruit (Fraser et al., 1999). Two functionally and structurally similar enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) catalyse phytoene to lycopene via ζ-carotene. Cyclization of lycopene result in production of series of carotenes that contain one or two rings of β or ε rings (see Figure 1-5). The ε lycopene cyclase (LCY-E) and the β-lycopene cyclase (LCY-B) add ε and β rings to lycopene respectively. Both of cyclases require the cofactor NADPH (Fraser and Bramley, 2004). In tomato, a β–lycopene cyclase (CYC-B) ,which is a chromoplast specific enzyme, has also been identified (Ronen et al., 2000). Consequently the first xanthophylls of lutein and zeaxanthin produced from α-carotene and β–carotene by introducing the oxygen atoms as hydroxyl groups. This process is catalysed by the carotene β-hydroxylase 1 and 2 (CRTR-B1 and CRTR-B2) and/or by the heme-containing cytochrome P450 β-ring hydroxylase (CYP97A) . The ε rings is introduced by a heme-containing cytochrome P450 ε-ring hydroxylase (CYP97C) (see Figure 1-5) (Tian and Dellapenna, 2001; Sun et al., 1996; Tian et al., 2004; Kim and DellaPenna, 2006). Zeaxanthin convert to antheraxanthin and then violaxanthin by the introduction of epoxy groups by Zeaxanthin epoxidase (ZEP-1) enzyme, which the reverse steps process by violaxanthin de-epoxidase (VDE-1) enzyme (Demmig-Adams and Adams, 1996).This is known as the xanthophyll cycle, which used in photo-oxidation stressed as explained in section 1.5.1.1.1. In the last reaction neoxanthin synthase (NXS) convert violaxanthin to neoxanthin (see Figure 1-5).

During ripening mRNA levels of PSY-1 and PDS enhanced considerably at breaker stage (Fraser et al., 1994). And also the mRNAs of both lycopene cyclases (LCY-B and LCY-E) decreased (Pecker et al., 1996; Ronen et al., 1999). Inhibition of lycopene cyclization in tomato leaves result in elevation in expression of both PDS and PSY-1 (Giuliano et al., 1993). This shows that transcriptional regulation plays a role in the accumulation of lycopene in the steps of fruit development ripening.

1.5.1.1.2.2 Ketocarotenoid biosynthesis pathway in bacteria

Ketocarotenoid are mostly biosynthesised in microorganisms. β-carotene is the first carotenoid precursor of the ketocarotenoid production. As represented in Figure 1-5 the responsible enzyme for production of geranylgeranyl diphosphate (GGPP) from farnesyl diphosphate (FPP) is bacterial geranylgeranyl diphosphate synthase (CRTE). bacterial phytoene synthase (CRTB) produce phytoene by condensation of two GGPP and bacterial phytoene desaturase (CRTI) bypass 4 plant enzyme actions by converting phytoene to lycopene (see Figure 1-5). The production of β-carotene is preceded by the lycopene cyclase (CRTY) (Misawa et al., 1990, 1995b). β-carotene by two steps of ketolation and two steps of hydroxylation convert to astaxanthin, which is the final production of β-carotene hydroxylases (CRTZ) and β-carotene ketolases (CRTW). Eight intermediates ketocarotenoids between β-carotene and astaxanthin are illustrated in Figure 1-6 (Fraser et al., 1997, 1998, Misawa et al., 1990, 1995b, 1995a; Zhu et al., 2009). *In vitro* characterisation of CRTZ and CRTW indicates that these enzymes need $O_2$ and $Fe^{2+}$ is required for optimal activity (Fraser et al., 1997).

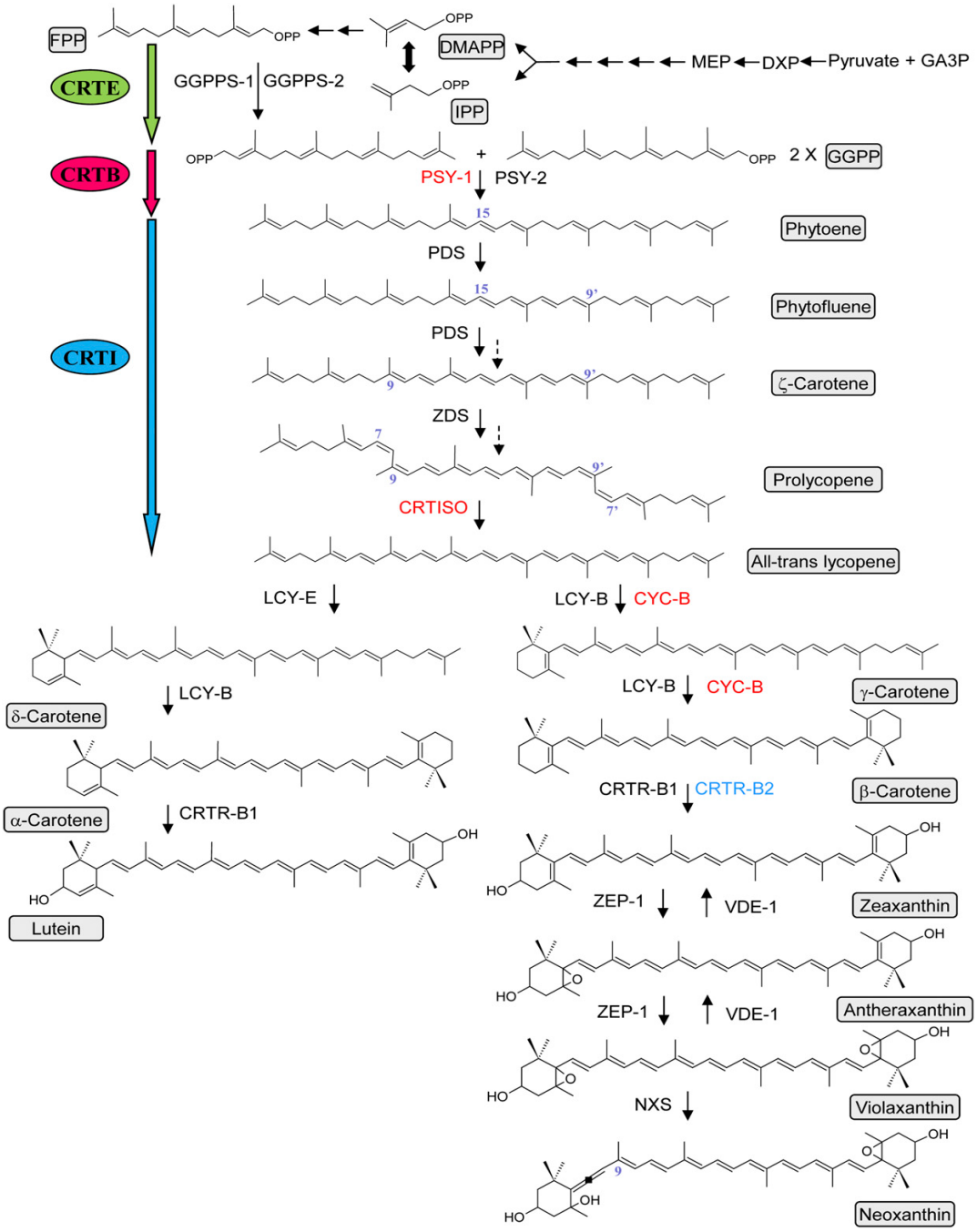



Figure 1-5 Scheme representation of the carotenoid biosynthetic pathway in higher plants and the effective bacterial genes (Nogueira et al., 2013)

"Enzymes in red are tomato fruit ripening specific or enhanced, and those in blue are flower specific. GA3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS-1 and -2, geranylgeranyl diphosphate synthase; PSY-1, fruit-specific phytoene synthase-1; PSY-2, phytoene synthase-2; PDS, phytoene desaturase; ZDS, δ-carotene desaturase; CRTISO, carotene isomerase; LCY-E, ε-lycopene cyclase; LCYB, β-lycopene cyclase; CYC-B, fruit-specific β-lycopene cyclase; CRTR-B1, carotene β-hydroxylase 1; CRTR-B2, carotene β-hydroxylase 2 (flower specific); ZEP, zeaxanthin epoxidase; NXS, neoxanthin synthase; VDE, violaxanthin deepoxidase; CRTE, bacterial geranylgeranyl diphosphate synthase; CRTB, bacterial phytoene synthase; and CRTI, bacterial phytoene desaturase. The *cis* configurations are not shown for all molecules, but they are represented with blue numbers. The dashed arrows illustrate biochemical steps that are not represented in this scheme."

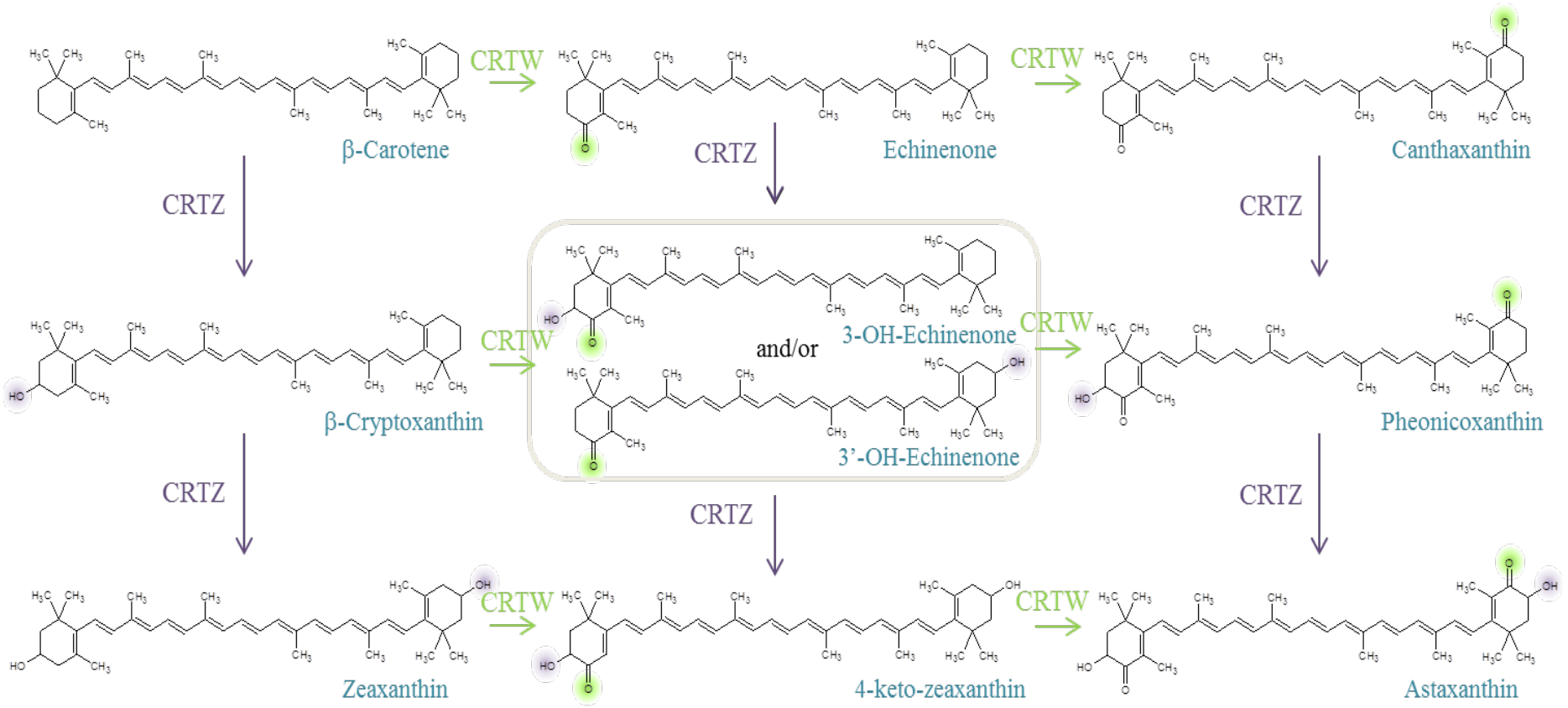



Figure 1-6 The ketocarotenoid biosynthetic pathway in bacteria (Nogueira, 2013)

"CRTZ, β-carotene hydroxylase; CRTW, β-carotene ketolase. Purple and green shadings indicate the position of newly introduced hydroxyl and keto groups, respectively.

#### 1.5.1.2 Fruit taste and aroma

##### 1.5.1.2.1 Tomato taste

Tomato taste is a result of a balance between sugars, organic acids (Stevens et al., 1997). Reduced sugars (glucose and fructose) and sucrose are the main sweetness factors in tomato. Sourness is because of organic acid content, mainly malic and citric acids. High sugars and acids are necessary for having a good flavour and are broadly accepted by consumers (Jasionowicz, 2012). Citric acid is the main organic acid in ripe tomatoes. The content of this acid during maturation increases (Apolinario et al., 1954).

##### 1.5.1.2.2 Tomato aroma

Volatiles are compounds with very low molecular weights and consequently evaporate quickly at room temperature. There are three sources for tomato volatiles, which are amino acids, carotenoids and lipids (Goff and Klee, 2006). Over 400 volatile compounds are known in tomato, but not a single compound is known to be directly related to tomato flavour (Buttery and Ling, 1993). Some aldehydes, ketones and some alcohols are the main flavours in tomato, which are synthetized in plant tissues during ripening. Secondary volatile compounds (for instance β-ionone, isovaleronitrile and trans-2-pentenal) are produced in small quantities in intact cells, but are generated at higher rates during cell disruption by cutting, crushing and slicing (Goodenough, 1990). The process of lipid oxidation is the main biogenesis pathway for volatiles production in tomato after fruit softening (Boukobza et al., 2001; Boukobza and Taylor, 2002).

#### 1.5.1.3 Antioxidants

Plant antioxidants inhibit the accumulation of reactive oxygen species (ROS). The important ROS damaging tissues consist of superoxide, H2O2, hydroxyl radical and singlet oxygen (Buettner and Jurkiewicz, 1996). ROS produce in result of secondary messengers in hormone signal transduction and also as the products of cellular metabolism. Oxidative damage only occurs when antioxidants cannot limit ROS accumulation. In tomato fruit, photo oxidative damage mostly happens in the green epidermal and hypodermal cellular structures (Rabinowitch et al., 1982). This damage typically occurs in chlorophyll containing tissues at high temperatures (Retig and

Kedar, 1967; Rabinowitch et al., 1974). In fruit photo oxidative stress mainly happens with normal process of ripening and especially with fruit senescence (Blackman and Parija, 1928; Brennan and Frenkel, 1977). In addition to carotenoids for defending oxidative stresses, there are some other compounds in tomato with antioxidant property such as ascorbic acid activity in hydrophilic environments (aquatic conditions like cytosol and stroma) and tocopherols play role in hydrophobic environments (for example membranes and lipids).

Vitamin C (ascorbic acid) is one of the important organic acids produced in plants with reducing capacity and antioxidant potential (Davey et al., 2000). Ascorbic acid is a hydrophilic compound with best activity in this environment. Ascorbic acid has different functions of antioxidant, pro-oxidant, a reducing agent or a metal chelator, which depend on its place, concentration and other factors. In aqueous media and in the absence of metals, ascorbic acid reacts as an antioxidant, especially at higher concentrations (Arrigoni and De Tullio, 2002). As an antioxidant, ascorbic acid play role in the scavenging of reactive oxygen species (Smirnoff, 2000).

There are three reactions that ascorbic acid play role for defending the oxidative stress which comprise the Mehler–peroxidase cycle (Buschmann, 1999; Neubauer and Yamamoto, 1992), the ascorbate–glutathione cycle (Foyer et al., 1976) and xanthophyll cycles (Neubauer and Yamamoto, 1992, 1979) which are illustrated in Figure 1-7. In Mehler–peroxidase cycle, transferring electrons from water to O2 and again back to water deactivates ROS specially $O^{2\bullet-}$. In the ascorbate–glutathione cycle ascorbate peroxidases (APX) inhibits H2O2. APX requires ascorbic acid regenerating cycles that use electrons from NAD(P)H as reducing power (Foyer et al., 1976). Xanthophyll cycle was explained in section 1.5.1.1.2. In high light, the Mehler–peroxidase and xanthophyll cycles compete for ascorbic acid (Andrews et al., 2004). The ability of ascorbic acid to transfer two hydrogen atoms makes it very important and effective as a reducing agent and bio antioxidant. After giving two hydrogens, it is converted to dehydroascorbic acid with possessing of ascorbic acid activity because of its potential for receiving oxygen species from the oxidised lipids. Immediately after oxidation of dehydroascorbic acid, its rapid breakdown to ketones and aldehydes without any vitamin C activity happens. *In vivo* and *in vitro* studies have shown effect of ascorbic acid in protecting lipids from oxidation (Simon, 1992). Furthermore, it has been shown that plasma of people with low vitamin C levels contain higher amounts

of lipid peroxides to people with high vitamin C levels. Vitamin C also can affect cholesterol metabolism through the antioxidant effect (Fidanza et al., 1982).

Ascorbic acid also is shown to play role in signal transducing metabolites with low concentrations in plants for example restricting growth through hormone action and pathogenesis resistance proteins (Kiddle et al., 2003; Pastori et al., 2003). The main sources of ascorbic acid are fruit and vegetables. A good dietary source of them especially citrus fruits, berries, peppers and broccoli (USAD, 2016) could help improving health factors result by Vitamin C.

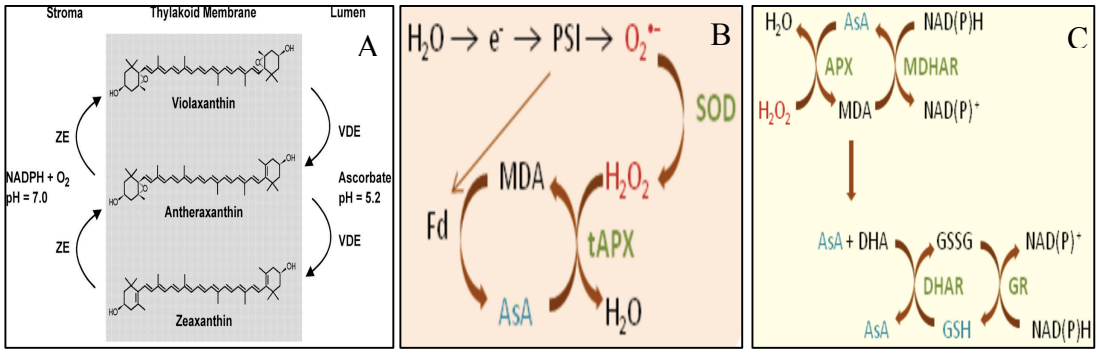



Figure 1-7 A: The violaxanthin or xanthophyll cycle in higher plants. B, the water–water cycle (Mehler reaction); C, the ascorbate–glutathione cycle (adapted from Mittler et al., 2004; Wituszynska and Karpinski, 2013).

(A) The cycle is organized transmembrane with stepwise de-epoxidation from violaxanthin to zeaxanthin by violaxanthin de-epoxidase (VDE) occurring on the lumen side and epoxidation by zeaxanthin epoxidase (ZE) on the stroma side. Light-induced acidification of the lumen to around pH 5.2 binds VDE to the thylakoid membrane. The epoxidase is bound to the thylakoid on the stroma side and catalyses epoxidation near neutral pH (Hieber et al., 2004; Yamamoto, 1979). (B&C) Superoxide dismutase (SOD) acts as the first line of defence converting O2•− into H2O2 and then ascorbate peroxidases (APX) eliminate H2O2. APX requires ascorbate (AsA) regenerating cycles that use electrons from the photosynthesis (B) or NAD(P)H (C) as reducing power. ROS are indicated in red, ROS-scavenging enzymes in green and low-molecular antioxidants in blue. Abbreviations: AsA, ascorbate; APX, ascorbate peroxidases; DHA, dehydroascorbate; DHAR, DHA reductase; Fd, ferredoxin; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, monodehydroascorbate; MDAR, MDA reductase; PSI, photosystem I; tAPX, thylakoid-bound APX.

As stated at the first of this section vitamin c is active in hydrophilic condition but tocopherols play role in hydrophobic environments. Tocopherols are the most frequently distributed antioxidants in nature. Considering their hydrophobic property, they act as the main antioxidant in vegetable oils. The small amounts of tocopherols observed in animal fats transferred from vegetable components in the animal diet. Different tocopherols present different types of Vitamin E. α-tocopherol is found to be the most biological effective member of vitamin E in human and animal organs

and the second commonly found vitamin E in diet (Fennema and Tannenbaum, 1996) Temperature and light considerably influence the relative activity of these compounds. As antioxidants, tocopherols present their maximum effectiveness at relatively low levels. Concentrations of α-tocopherol in biological membranes are approximately one molecule per 1000 lipid molecules (Burton et al., 1983). If tocopherols are used at high concentration, they might play role as pro-oxidants. Because α-tocopherol can compete for peroxy radicals considerably faster than polyunsaturated fatty acids, little amount of α-tocopherol can protect a large amount of polyunsaturated fats (Shalluf, 2010).

### 1.5.2 Important TFs and regulatory genes affecting ripening

Fruit ripening is a highly programmed mechanism. The discovery of transcription factors which play roles in controlling the switch to the ripening step were obtained mainly from map based cloning of genes responsible for rare natural non ripening mutations in tomato. These single gene mutations include *RIN* (ripening-inhibitor), *CNR* (colourless non ripening) and *NOR* (non ripening), and their locus encoding *MADS-box* (conserved sequence motif), *SBP-box* (squamosa promoter binding protein-box) and *NAC* (conserved sequence motif) transcription factors , respectively. Mutations in these TF genes prevent fruit ripening steps, such as colour development, softening and the climacteric increase in respiration rate and ethylene production (Giovannoni, 2007; Manning et al., 2006; Vrebalov et al., 2002; Eriksson et al., 2004).These TFs are affecting upstream of ethylene-dependent and independent ripening regulatory pathways and also regulating the expression of many ripening related genes directly and indirectly (Giovannoni, 2007). *RIN* belongs to a subfamily of *MADS box* TFs named *SEPALLATA* and *RIN* express under a ripening specific manner (Vrebalov et al., 2002). *RIN* mutant represent unripe fruits with alteration and inhibition in expression of many ripening-related genes (Fujisawa et al., 2012). Over 240 target genes were identified to play role in many aspects of fruit ripening for instance ethylene synthesis and signalling, controlling other ripening related TFs like *NOR*, *CNR*, *TDR4* (*FRUITFULL homologue*) and (*HD-ZIP HOMEBOX PROTEIN-1*) *HB-1*, carotenoid accumulation, cell wall modification and aroma production (Fujisawa et al., 2013; Qin et al., 2012; Martel et al., 2011). Studying on the downstream TFs of *RIN* is considered by many scientists nowadays for finding new TFs that affect ripening.

In addition, several TF genes, including *APETALA2a* (*AP2a*), *AGAMOUS-LIKE1* (*TAGL1*), *HD-ZIP HOMEBOX PROTEIN-1* (*HB-1*) and two FRUITFULL homologs (*TDR4* and *MBP7*), have been found to play significant roles in fruit ripening in tomato (Lin et al., 2008a; Thompson et al., 1999; Vrebalov et al., 2009, 2002; Fujisawa et al., 2013; Lee et al., 2012; Chung et al., 2010).

*AP2a* silenced fruits represent the role of *AP2a* during tomato fruit development and ripening, controlling chromoplast differentiation, regulation of primary and secondary metabolites, signalling pathways and ethylene biosynthesis. *RIN* and *CNR* ripening regulators function upstream of *SlAP2* and affects its expression positively. It is shown that *CNR* binds to the promoter of *SlAP2a* directly *in vitro*. In the *AP2a* transgenic fruits some of ripening-related such as carotene biosynthesis pathway and pectin methylesterase were downregulated; showing that *AP2a* affects positively on ripening regulatory functions besides its negative regulatory function in ethylene biosynthesis (Chung et al., 2010; Karlova et al., 2011).

*TAGL1* interacts with *RIN* (Leseberg et al., 2008) and were shown to be highly expressed during fruit ripening (Itkin et al., 2009; Vrebalov et al., 2009). It is reported that *TAGL1* functions as a positive regulator in fruit development and ripening (Itkin et al., 2009; Vrebalov et al., 2009). *TAGL1* knockdown plants result in yellow/orange fruits with thin pericarp and reduced carotenoids. They represent low ethylene levels in result of decreased expression of *ACS2*, and *TAGL*1, which are *RIN* targets. It seems that *RIN* and *TAGL1* work together for regulating ripening by inducing autocatalytic system 2 ethylene production (Itkin et al., 2009).

Another important TF affecting ripening is *LeHB-1*, which encodes a homebox protein binding ethylene biosynthesis gene promoter (*LeACO1*) in vitro. The *LeHB-1* silenced tomato fruits present reduction in *LeACO1* mRNA levels and inhibited ripening (Lin et al., 2008b; Karlova et al., 2014).

The *HIGH-PIGMENT* (*hp*) mutants in tomato other than their important effects on plant development may also have role in the ripening regulation. The *high pigment1* (*hp1*) and *hp2* mutations represent lesions in tomato orthologous locus of the Arabidopsis genes *UV-DAMAGED DNA-BINDING PROTEIN*-1 (*DDB1*) and *DE-ETIOLATED* 1 (*DET1*). Both of mutants indicate high fruit carotenoid levels and pigmentation (the main alteration in tomato fruit ripening is a peak in production of carotenoids and pigmentation), and the gene products take part in the suppression of light responses in absence of light (Mustilli et al., 1999; Enfissi et al., 2010). The

translated proteins of these two genes form a complex with *Cullin4* (*CUL4*) (an ubiquitin-conjugating E3 ligase). This complex targets proteins for proteolysis. Down regulation of *CUL4* also enhanced fruit pigmentation (Wang et al., 2008). *DET1* does not seem to participate in the regulation of proteolysis. It is suggested that *DET1* acts through chromatin remodelling by binding to nucleosome core particles via interacting with the N-terminal tail of histone H2B. Preferentially *DET1* binds to non-acetylated H2B tails which suggest *DET1* binds to the nucleosomes of light-inducible genes and results in light dependent acetylation of the histone tails and finally permits the process of gene expression (Benvenuto et al., 2002). However, the effects of the various hp mutations are not fruit specific. The fruit specific transgenic constructs of down regulated *DET1* result in elevation of plastid area and numbers also enhancement of other metabolites such as carotenoids without negative effects on vegetative tissues (Davuluri et al., 2005; Enfissi et al., 2010).

*GARP* family transcription factors [contain *GLK* sequence] belonging to *Myb* TFs are another important TFs affecting pigmentation and ripening. They have shown an effect for increasing chloroplast formation by light stimulation. *GLK2-like* transcription factor in tomato affects carotenoids and chlorophyll biosynthesis (Powell et al., 2012; Fitter et al., 2002). *APRR2-Like* transcription factor [*ARABIDOPSIS PSEUDO-RESPONSE REGULATOR-2*] have a high homology with *GLK-Like* genes. *APRR2* gene consists of a *GARP* DNA binding domain and a receiver-Like domain in the N terminus. But in Arabidopsis *APRR2* lacks an important motif which is a conserved motif (AREAEAA) in *GLK* genes, therefore *APRR2* is known distinct from this group (Fitter et al., 2002; Pan et al., 2013). In tomato *APRR2-Like* is shown that affect the accumulation of pigments in fruit plastid, increase plastid numbers, areas and improving the levels of chlorophyll in immature unripe fruits as well as carotenoids in red ripe fruits. Other than enhancing pigment levels, increase in transcript level of *LeMADS-RIN*, *ACO*, and *PG* genes shows its broad effective area (Pan et al., 2013).

### 1.5.3 Ethylene signalling genes

Ethylene is a very important and well-studied hormone promoting the ripening. Climacteric ethylene induces and promotes many alterations such as pigment accumulation and fruit softening. Two systems of ethylene production were identified in climacteric plants, which are illustrated in Figure 1-11. System 1 relates to normal

growth and development stages and also during stress responses, while system 2 react during floral senescence and fruit ripening. The first system is an auto inhibitory system. Exogenous ethylene inhibits its endogenous biosynthesis, and inhibitors of ethylene application can stimulate ethylene production. In contrast, system 2, which is an autocatalytic system, is stimulated by exogenous ethylene, and ethylene inhibitors inhibit the ethylene production (McMurchie et al., 1972; Barry and Giovannoni, 2007). The autocatalytic increase in respiration and ethylene biosynthesis occurs just prior to the initiation of ripening. Ethylene signalling can be regulated at different levels, which include ethylene biosynthesis and its conjugation with different ethylene receptors encoded by *ETHYLENE RESPONSE* (*ETR*) genes.

Tomato genome contain seven ethylene receptor genes (*LeETR1, LeETR2, NR, LeETR4, LeETR5, LeETR6, and LeETR7*) (Lashbrook et al., 1998; Tieman and Klee, 1999; Wilkinson et al., 1995). Five of these receptors represented binding to ethylene and *LeETR6* and *LeER7* were not tested yet (O'Malley et al., 2005). Ethylene receptors based on gene and protein structures are divided into subfamily 1 (*LeETR1, LeETR2, NR, LeETR5, and LeETR7*) and subfamily 2 (*LeETR4, LeETR6*). Reduced expressions in the subfamily 2 receptor genes, *LeETR4* or *LeETR6*, represent considerable increase in ethylene sensitivity. Also antisense downregulated plants enhanced ethylene sensitivity and significantly earlier fruit ripening (Kevany et al., 2007; Tieman et al., 2000). This increased ethylene sensitivity reported to be restored to wild type phenotype by overexpression of *NR* from subfamily 1 receptor. Three receptors of *LeETR4, LeETR6,* and *NR* expression elevated significantly at the onset of fruit ripening. These three receptor genes are the most expressed genes in ripening fruits. In tomato the NR (never-ripe) mutation blocks ethylene perception. Loss of function for any of the other receptors did not indicate any affect on ethylene sensitivity or ripening pattern (Kevany et al., 2007). Together these results show that the subfamily 2 receptors are more important functions in tomato (Klee and Giovannoni, 2011).

ETRs activate a signal transduction cascade at first step by *CONSTITUTIVE TRIPLE RESPONSE 1* (*CTR1*) and consequently via *ETHYLENE INSENSITIVE 2* (*EIN2*). This release at next step activates *EIN3/EIN3-Like* (*EIL*) primary TF genes (Tieman et al., 2001). The expression of secondary transcription factors, ethylene response factors or *ERF*s happened in result of *EIL* activation and regulation of target gene expression is the final step of signalling is by *EIL*s or *ERF*s (Karlova et al., 2014).

Several genes play role in tomato ripening through ethylene signal transduction are identified. Some of genes are encoding the ethylene receptor genes such as *NEVER-RIPE* (*NR*) (Wilkinson et al., 1995; Yen et al., 1995), *ETR6* (Kevany et al., 2007), and *GREEN-RIPE* (*GR*, an unknown protein function) (Barry et al., 2005; Barry and Giovannoni, 2006). The *ERF* genes from the large *AP2/ERF* family mediate ethylene dependent gene expression via binding to specific GCC motif in promoter region of target genes (Pirrello et al., 2012). It is shown that *LeERF1* positively mediates the ethylene signalling in tomato fruits and also in seedling. *ERF1* RNAi fruits indicate longer shelf life compared with normal fruits. Another member of the *ERF* family, *Sl-ERF.B3*, has been shown to act as a transcriptional activator on GCC box-containing promoters (Liu et al., 2013). Down regulated RNAi *SlERF6* increased both carotenoid and ethylene levels during ripening, indicating the important role of *SlERF6* in fruit ripening (Lee et al., 2012). The molecular regulators effecting ripening illustrated in Figure 1-8.

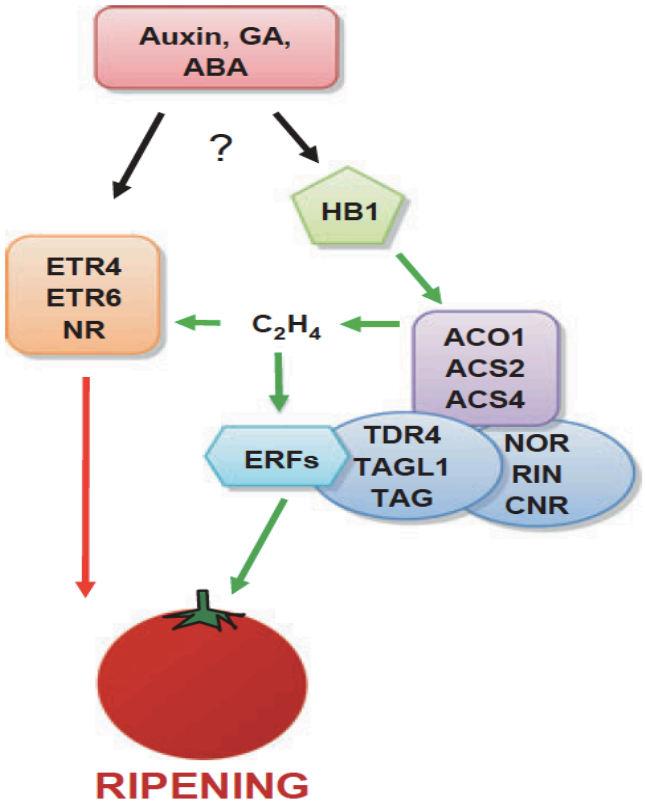



Figure 1-8 Molecular regulators of ripening in tomato fruits.

"Ethylene biosynthesis is triggered by HB1 and other developmental cues and also promoted by ripening-related transcription factors, for example RIN and TAGL1. Ethylene perception by receptors

encoded by members of the ETR and NR gene family results in receptor degradation and ethylene signal transduction. This initiates and coordinates the ripening process" (Seymour et al., 2013a).

### 1.5.4 ABA biosynthesis

ABA biosynthetic pathway is well studied in higher plants (Figure 1-9) and also many mutants have been characterised for each step in this pathway for tomato (Burbidge et al., 1999; Nitsch et al., 2009), Arabidopsis (North et al., 2007; Huo et al., 2013) and maize (Tan et al., 1997, 2001). *De novo* synthesis of ABA started from a C40 carotenoid. The carotenoid biosynthesis pathway starts with phytoene formation from two molecules of geranylgeranyl diphosphate (GGPP) in the isoprenoids pathway. Four desaturation steps result in lycopene synthesis which with two cyclization at both ends of lycopene $\alpha$ or $\beta$ carotene are produced and two hydroxylation at C3 and C3′ produce lutein and zeaxanthin respectively form the xanthophylls family. Detailed pathway of carotenoids illustrated in section 1.5.1.1.2. With β-carotene hydroxylation and epoxidation an important phase of ABA biosynthesis is started in plastids to produce the zeaxanthin and violaxanthin by ZEP enzyme. Violaxanthin is then altered to 9-cis-epoxyxanthophylls and after that oxidative cleaved by 9-cis-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin, which is the first C15 intermediate of ABA synthesis; xanthoxin exits the plastid and transported to the cytosol where undergo two further oxidization steps to form ABA (Qin and Zeevaart, 1999; Schwartz et al., 2003; Taylor et al., 2005).

Overexpression experiments in transgenic plants are indicated that NCED is a key-limiting step in regulating the rate of ABA biosynthesis (Iuchi et al., 2001; Thompson et al., 2000; Tung et al., 2008). Minimum of four *NCED* genes were cloned to date in tomato fruits (Huo et al., 2013). ABA levels in tomato fruit are controlled predominantly by *SlNCED1* at the transcriptional level and moreover in response to environmental stimuli such as dehydration (Zhang et al., 2009). Tomatoes overexpressing *SlNCED1* had increased ABA accumulation and drought tolerance as well as reduced levels of transpiration (Thompson et al., 2000). An ABA deficient tomato mutant represented mean fruit weight reduction and did not indicate the normal development and ripening in comparison to wild type (Galpaz et al., 2008). Also, in a silenced transgenic *SlNCED1*-RNAi tomato construct reduction in endogenous ABA levels result in inhibited cell wall degradation showing that ABA is

essential during fruit development and ripening (Sun et al., 2012a, 2012b). Overexpressed genes responsible for regulatory enzymes in the methylerythritol phosphate (MEP) pathway such as DXS and xanthophyll cycle (ZEP) can increase ABA accumulation in seeds or seedlings (Frey et al., 1999; Estévez et al., 2001; Lindgren et al., 2003). Considering all points together, it can be concluded that regulation of ABA metabolism is not just restricted to defined steps in ABA production (NCED and CYP707A) but is also regulated with other pathways that are upstream of ABA biosynthesis (Leng et al., 2014).

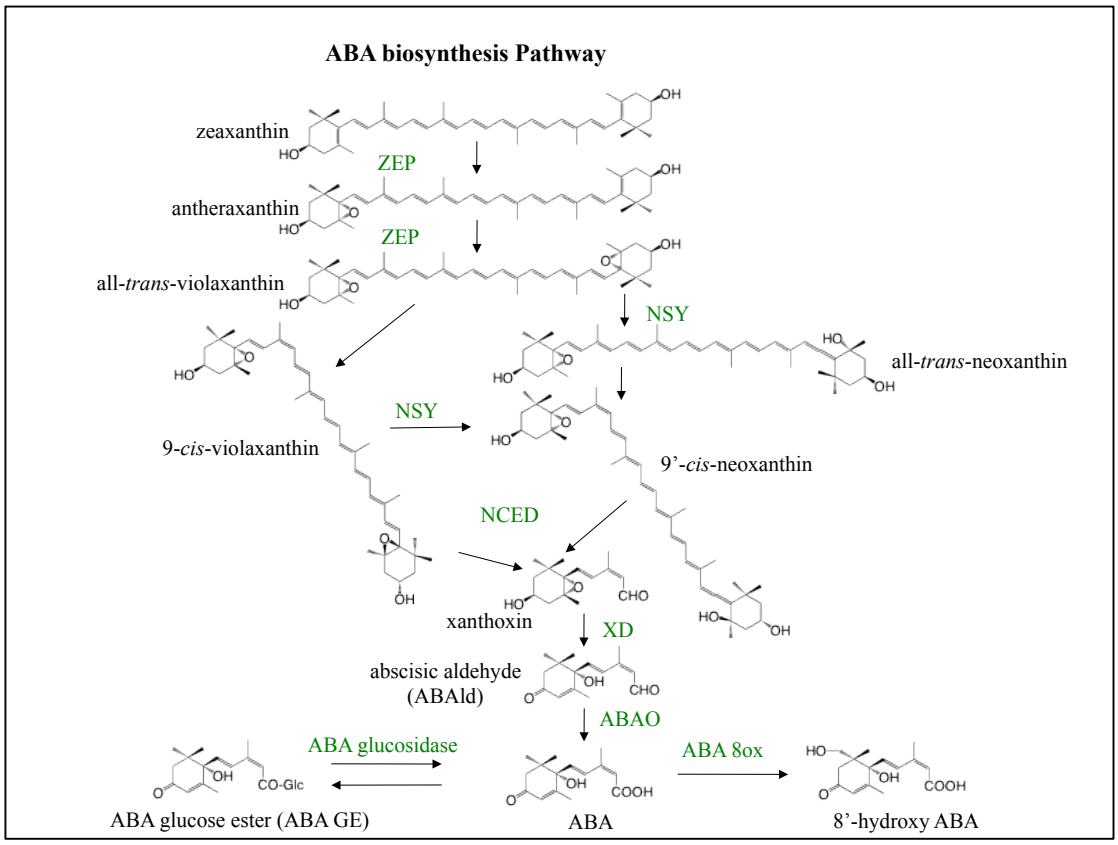



Figure 1-9 Abscisic acid biosynthesis pathway (adapted from http://hormones.psc.riken.jp/pathway_aba.shtml)

ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxigenase; XD, xanthoxin dehydrogenase; ABAO, abscisic aldehyde oxidase; ABA8ox, ABA 8'-hydorxylase.

### 1.5.5 Ethylene biosynthesis

The ethylene biosynthesis pathway in higher plants is well studied. Briefly, ethylene biosynthesis starts from methionine in three steps:

(1) SAM synthase enzyme converts methionine to S-adenosyl-L-methionine (SAM).

(2) 1-aminocyclopropane-1-carboxylic acid (ACC) forms from SAM by ACC synthase (ACS) activity

(3) Conversion of ACC to ethylene catalysed by ACC oxidase (ACO) (see Figure 1-10).

Formation of ACC also results in production of 5'-methylthioadenosine (MTA). This compound recycled via the MAT cycle to a new molecule of methionine.

Increased respiration of system II, provides the ATPs which is needed for methionine cycle and could produce high rates of ethylene production without need to high levels of intracellular methionine (Alba et al., 2005; Zegzouti et al., 1999).

*ACS* and *ACO* are encoded by multi gene families in tomato which encodes at least nine *ACS* genes (*LEACS1A, LEACS1B,* and *LEACS2-8*) and five *ACO* genes (*LEACO1-5*) (Barry et al., 1996; Nakatsuka et al., 1998; Oetiker et al., 1997; Van der Hoeven et al., 2002; Zarembinski and Theologis, 1994). The biosynthesis pathway of ethylene with the transcriptional regulation of this pathway is illustrated in Figure 1-10.

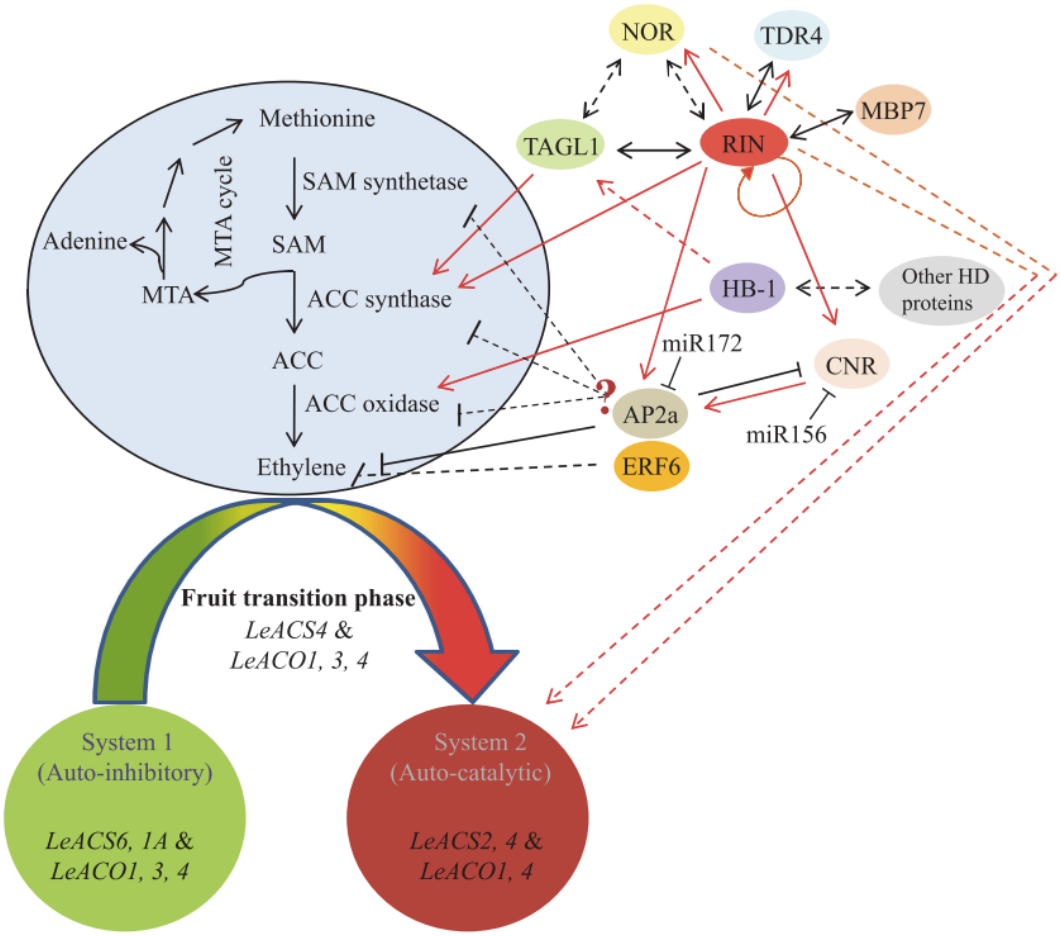



Figure 1-10 "Regulation of ethylene biosynthesis during fruit development and ripening" (Kumar et al., 2014).

"Upper part of the figure shows the regulation of various ethylene biosynthesis steps by different developmental regulators during ripening in tomato. The lower part of the figure shows the transition phase from green to red tomato fruits and participation of *ACS* and *ACO* genes in different phases of fruit development. Dotted lines/arrows represent the proposed but not yet experimentally confirmed interactions among the genes/proteins. Solid lines/arrows show the interactions supported by some experimental findings. Bidirectional black arrows indicate protein–protein interactions between the two ripening regulators. Solid red arrows denote the interactions between ripening regulator proteins and their target promoters. Red dotted arrows indicate that *RIN* and *NOR* regulate fruit ripening primarily via controlling the ethylene biosynthesis aspect during system 2. (⊥) sign denotes negative regulation of these genes by their regulators. Abbreviations: SAM, S-adenosyl- methionine; SAMS, SAM synthase; ACC, 1-aminocyclopropane-1-carboxylate; ACS, ACC synthase; ACO, ACC oxidase; SAMDC, SAM decarboxylase; *NOR*, non-ripening; *TAGL1*, tomato *AGAMOUS-Like 1*; *RIN*, ripening-inhibitor; *TDR4* and *MBP7*, tomato *FRUITFULL* homologs; *HB-1*, homeobox protein-1; *CNR*, colourless non-ripening; *AP2a*, AP2 domain containing ethylene response factor (*ERF*); and *miR156* and *172*, microRNA 156 and 172, respectively" (Kumar et al., 2014).

### 1.5.6 Hormonal regulation of ripening

The process of ripening consists of multiple genetic and biochemical pathways.

As these changes detected in the context of response of different hormones, the main hormones mediating ripening are predominantly ethylene and ABA (Giovannoni, 2004; McAtee et al., 2013). There is a considerable increase in ABA level during ripening in fleshy fruits. It is shown that any treatment, which delays ABA elevation, result in delay the induction of ripening (Zhang et al., 2009). In tomato and peach fruits a peak in ABA level precedes the climacteric ethylene production. It is indicated that ABA induce ripening by promoting ethylene biosynthesis by upregulation of ethylene biosynthesis genes (Sun et al., 2012c).

Predominant role of ethylene in ripening process of the climacteric fruits, makes ethylene the most explored hormone (Bapat et al., 2010). Two systems of ethylene biosynthesis react during fruit development and ripening in all climacteric fruits. Ethylene is produced at very low level in system 1 and is autoinhibitory but ethylene production considerably elevated in system 2 during ripening and is autocatalytic (Van de Poel et al., 2012). The ethylene production in the two systems is controlled by regulation of various ACC synthase (*ACS*) and ACC oxidase (*ACO*) genes (see Figure 1-11) (Barry et al., 2000). In climacteric fruits, several evidences suggest ethylene and indole-3-acetic acid (IAA) are in crosstalk with each other in system 2 during ripening which supported by two observations that (i) simultaneous increase of IAA and ethylene is observed in tomato and peach fruits and (ii) genes responsible for ethylene biosynthesis (*ACS2*, *ACS4* and *ACO1* etc.) and ethylene signalling (*ETR*s and *ERF*s etc.) were upregulated by auxin and vice versa (see Figure 1-11 & Figure 1-8) (Gillaspy et al., 1993; Wilkinson et al., 1997, 2012; Jones et al., 2002; Trainotti et al., 2007).

In addition, gibberellin hormone (GA) has also been shown to delay fruit ripening in many fruits for instance tomato, peach, mango, sapota, etc. (Dostal and Leopold, 1967; Martínez-Romero et al., 2000; Singh et al., 2007; Sudha et al., 2007). In addition to these hormones, endogenous level of methyl jasmonate (MJ) elevated with progress of ripening in tomato, apple, pear and mango (Fan et al., 1998; Kumar et al., 2014).

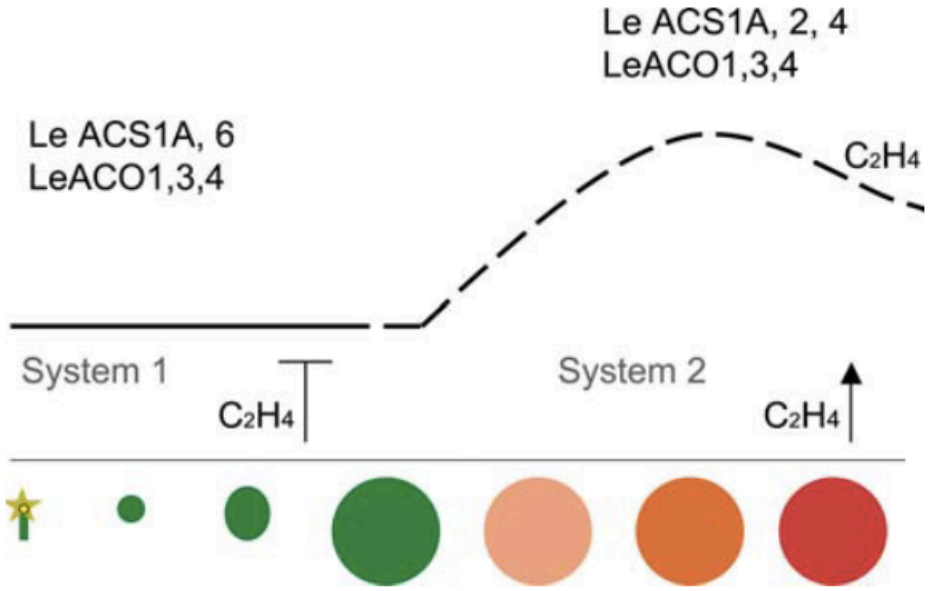



Figure 1-11 System I and II in ethylene production.

"Differential expression of *ACS* and *ACO* genes associated with system 1 and system 2 ethylene synthesis during fruit development and ripening in tomato. Autoinhibition of ethylene synthesis during system 1 ethylene production is mediated by a reduction in *LeACS1A* and *6* expressions. Autocatalytic ethylene synthesis at the onset of fruit ripening is mediated through ethylene-stimulated expression of *LeACS2* and 4 and *LeACO1* and 4" (Barry and Giovannoni, 2007).

### 1.5.7 Cell wall alterations during ripening

#### 1.5.7.1 Cell wall structure

Plant cell hardness and firmness is needed for supporting the plant and providing the desired shape for tissues and fruits. This important means is achieved by the cell wall, which is an important part of plant cells. Primary plant cell wall is a complex macromolecular network containing different structural polymers. Plants cell wall consist of hemicellulose, pectin, cellulose, and protein and starch (Huber, 1983b; Fischer and Bennett, 1991). The ratios of these macromolecules varies based on species and stages of ripening (Lunn et al., 2013). Considering the formation of new cells mainly occur close to the apex, the young cells are exposed to less mechanical stress in comparison for example stem section. The cell wall composition in fruit tissue and young tissues is mainly constituted of cellulose, hemicellulose and pectin, which is the primary cell wall. Except xylem vessels, the fruit cells just have the primary cell walls. The cell wall directly controls the rate and direction of cell growth

and maintaining cell-cell adhesion. The cell wall conduct the direction and rate of cellular growth and also is responsible for maintaining the turgor pressure. In non-fruit tissue and structural zones secondary cell walls provide additional protection, rigidity and strength to. These walls are constructed of layers of cellulose micro fibrils, which the fibres are located in parallel with each layer. Addition of lignin to this structure result in less flexibility and makes the secondary cell wall less permeable to water than the primary cell wall (Raven and Evert, 1999; Lunn et al., 2013). The cell wall composition of tomato fruits will be explained in the next sections (Tibbits, 2000) .

#### 1.5.7.1.1 Cellulose

Cellulose consists of linear polymer of l–›4 D-glucose. The cellulose molecules conformation result in capability of formation of hydrogen bonds and forming the polymer called microfibrils. The diameter of each microfibril is nearly 8 to 10 nm. Cellulose forms 30% of cell wall component (see Figure 1-12) (Tibbits, 2000).

#### 1.5.7.1.2 Hemicellulose

In of dicotyledons the major hemicellulose in primary cell wall is xyloglucan. Xyloglucan forms about 20% of primary cell wall. The basic structure of xyloglucan consist of 1–›4 linked β-glucose substituted backbone with 1–›6 α-D-xylose links as side chains (Darvill and Albersheim, 1984; Fry, 1986; Cosgrove et al., 2002).

#### 1.5.7.1.3 Pectin

Pectins consist of alternating parts of chemically distinct blocks. Three distinct segments are known as homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII). HG forms from linear polymer of 1–›4 linked α-D-galacturonic acid (GalA). The HG polymer is chained by 1–›2 linked α-L-rhamnose residues (Rha), which apparently links within the polymer (Jarvis, 1984; Grant et al., 1973). The lengths of the HG regions determine the gelling properties of pectins. GalA-GalA linkages are much more stabled in acidic environment than GalA-neutral sugar (Rha) linkages. Therefore at low pH it is possible that the GalA-Rha bonds would be hydrolysed and consequently determines the lengths of unhydrolysed HG (Powell et al., 1982). RGI is a region of pectin containing high ratio of Rha:GalA and neutral sugars specifically D-galactose, L-arabinose and L-fucose. RGIl is a complex region known for existence of many different sugars. Quantification of total number

of sugar residues in RGIl is determined around 30 residues (Ishii et al., 1999). It seems the variation in the sugar composition of RGIl among species is low, and also even between groups of plants (see Figure 1-12) (Thomas et al., 1987).

#### 1.5.7.1.4 Cell wall proteins

Along with structural polysaccharides a number of proteins also are detectable in the cell wall. Extracellular proteins are divided into two groups of structural proteins and enzymes. The cell wall contain mainly catalytic enzymes such as polygalacturonases, galactanases, cellulases, glucanases, and other polysaccharide modifying enzymes, for instances pectin methyl esterases and xyloglucan endo-transglycosylase (Fry, 1995). A vast number of structural proteins also are detectable in the cell wall; Most of characterised structural proteins contain particular amino acid motif with a high degree of repetition (Cassab, 1998). The structural proteins seems to play one of mechanical or regulatory roles, for instance extensions play role in cell wall polymer adhesion (Fry, 1986) and expansions are expressed during ripening, indicating role of this proteins in fruit softening (Cosgrove et al., 2002; Rose et al., 1997). The abundance of different types of structural proteins is different in various cell types and also they localised specifically based on their structural roles. Whatever the role of the structural proteins, the location would appear to be specific to their function (Tibbits, 2000).

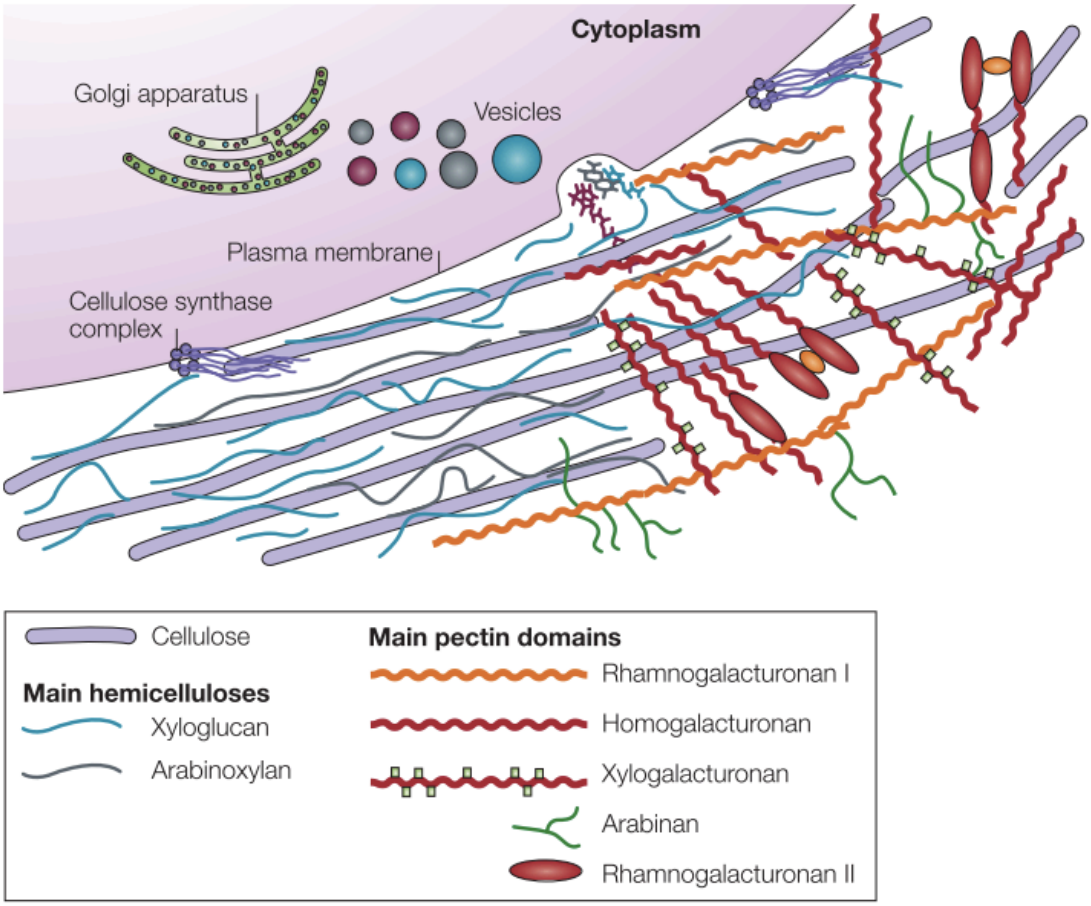



Figure 1-12 Structure of the primary cell wall (Cosgrove, 2005).

"Cellulose microfibrils (purple rods) are synthesized by large hexameric complexes in the plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles. For clarity, the hemicellulose– cellulose network is shown on the left part of the cell wall without pectins, which are emphasized on the right part of the figure. In most plant species the main hemicellulose is xyloglucan (blue), while hemicelluloses such as arabinoxylans (grey) and mannans (not shown) are found in lesser amounts. The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I (not shown) and rhamnogalacturonan II. Pectin domains are believed to be covalently linked together and to bind to xyloglucan by covalent and non-covalent bonds. Neutral pectin polysaccharides (green) are also able to bind to cellulose surfaces." (Cosgrove, 2005).

### 1.5.7.2 Cell wall degrading enzymes and fruit softening

Fruit ripening result in a series of biochemical alterations that change the unripe hard, green, acidic tissue into soft texture, colourful appearance, sweet taste and good aroma. Cell wall disassembly results in changes in fruit softening during ripening (Brummell, 2006). Cell wall disassembly during ripening conduct mainly by the activity of secreted polysaccharide-modifying enzymes into the cell wall space.

Moreover the non-enzymatic mechanism also can play role by the action of free radicals (Dumville and Fry, 2003). One of the main factor results in cell separation and fruit softening is solubilisation of the middle lamella pectin. The ratio of conversion of water-soluble pectin to chelator extractable pectin enhanced through ripening (Redgwell et al., 1991). Also considering two point that calcium chelators are able to proceed cell separation and the ability of pectin degrading enzymes for following the fruit ripening and tissue softening have a prominent role in the process of ripening. Different enzyme affecting pectin degradation during ripening could be named as the endo-polygalacturonases, the pectin methyl esterases and galactanase.
A family of expansins also are expressed during ripening and are take part in pectin depolymerisation and middle lamella solubilisation (Brummell et al., 1999a, 1999b)

#### 1.5.7.2.1 Endo Polygalacturonase

Previously endo polygalacturonase (PG) was the most likely potential candidate for pectin solubilisation as *In vitro* PG activity results in a decrease in the molecular weight of pectin. It was suggested that PG breaks down pectin resulting in middle lamella solubilisation and consequently cell separation and the tissue softening. It was shown that RIN (Ripening Inhibitor) and NOR (Non Ripening) two ripening mutants have reduced rates of softening and both reduced PG expression and enzyme activity (Dellapenna et al., 1989). The RIN and NOR mutants changed back to the wild type phenotype by insertion of the PG gene controlling by an ethylene induced promoter (Giovannoni et al., 1989). Transgenic fruits with antisense suppression of PG represent 99% suppression of PG activity result in reduced pectin depolymerisation but no significant reduction in pectin solubilisation and rate of softening (Smith et al., 1990). And also a transgenic tomato with an insertion in the PG gene which caused PG gene inactivation represent fruits with normal softening (Cooley and Yoder, 1998). It was also shown that in some cases fruit with decreased PG activity present firmer fruits than controls (Brummell and Labavitch, 1997). It has become clear that the process of fruit softening is more complicated than what was assumed before and softening is not started by PG action (Tibbits, 2000). Therefore it is apparent that PG role is not as initiating of fruit softening, but is more active in facilitating the later stages of softening (Tibbits, 2000).

#### 1.5.7.2.2 Pectin Methyl-Esterase

Pectin Methyl-Esterase (PME) is expressed early during ripening and is detectable in 10-20 day old tomato fruit (Harriman et al., 1991). Removal of the methyl ester groups from the GalA backbone is done by PME (Markovič and Kohn, 1984). Antisense suppression of PME mRNA with reduced activity of this enzyme by 90% in tomato present little effect on fruit firmness (Tieman et al., 1992). Antisense PME fruits showed reduced shelf life (Thakur et al., 1996; Tieman et al., 1992). And also the PME activity in not softening NR and NOR mutants was similar to control tomato lines (Harriman et al., 1991). On the contrary to NOR and NR mutants, CNR with a firm non ripening fruits (Thompson et al., 1999) present an inactive PME isoform associated with ripening. Consequently represent reduction in cell to cell adhesion and long un-methylesterified homogalacturonan (HGA) was not detected in this mutant; In addition to the unesterified HGA, CNR also seems to contain different structure which may affect calcium binding and cell adhesion (Orfila et al., 2002).

#### 1.5.7.2.3 Galactanase

During tissue softening an increase in the level of soluble cell wall galactose was detected. Galactanase enzyme is responsible for galactan degredation (Tibbits, 2000). Seven tomato β-galactosidase genes (TBG) were identified that were expressed during ripening correspond to three isoforms of known galactanase enzyme (Smith and Gross, 2000). TBG1 antisense tomato plants resent 10% reduction in mRNA accumulation to normal levels, which did not affect total β-Gal level (Carey et al., 2001). TBG 1, 2, 3 and 5 were detected in RIN, NOR and NR mutants and the pattern of their expression were similar to wild type. Reduced levels of TBG3 resulted in lower activity of this enzyme, modified cell wall composition and also decreased rate of fruits deterioration. TBG4 transcript accumulation was significantly deficient in all three mutants in comparison to wild type, which implies that the TBG4 may be involved in cell wall modifications result in fruit softening. TBG6, was presented in mutants but not detected in wild fruits (Smith and Gross, 2000).

#### 1.5.7.2.4 Ripening Expansins

In the tomato fruit ripening process, a family of expansins proteins (Exp) are expressed; especially Exp1, Exp3 and Exp5. Exp1 is shown to be present in fruit until the breaker point and after that is detectable in very small quantities. Exp5 is expressed and present in flowers and fruit until mature green stage. But Exp3 is

expressed later at the mature green stage and is highly detectable until the overripe stage. This protein reaches its peak at the pink and light red stages (Brummell et al., 1999b). Down-regulation of the Exp1 to 3% of wild type present increased fruit firmness and decreased pectin level but did not present any change in hemicellulose depolymerisation during fruit development. On the other hand over-expression of Exp1 presented softer fruit and increased pectin depolymerisation (Brummell et al., 1999a). From these observations it was suggested that Exp1 and probably other expansions affect pectin and middle lamella solubilisation in the early stages of fruit softening but do not have effects on hemicellulose degradation (Brummell et al., 1999a).

The recent researches result in conclusion that ripening is a complex process and multiple cell wall enzymes are play role for changing in texture during ripening. It is a complex action between various genes and different enzymes (Seymour et al., 2013a, 2013b).

## 1.6 Fruit over ripening

As soon as ripening started, it cannot be stopped and consequently can lead to over ripening. This process affects negatively on fruit quality parameters and its susceptibility to pests, which finally result in fruit discarding (Klee and Giovannoni, 2011; Seymour et al., 2013b). Although there is not a clear definition available for fruit over-ripening but it could be defined as programmed senescence of tomato fruit. In over ripening process the fully ripe tomato fruit begin some alterations, which shorten their shelf life (Zhang, 2013). The phenotypic changes of over ripening stage consisted of over-softening, loss of flavour and infection by post-harvest pathogens. The result of over ripening turns fruits to inconsumable product and consequently its discarding. Over-ripening process can be count as determined process for some fruits such as tomato. As the seeds hidden inside intact fruit and no proper seed dispersers are available, the plants have to use an alternative strategy for releasing their seeds. For instance in tomato fruit, as the seeds are located inside the fruit, the breakdown of fruit for seeds release seems necessary. Thus the decaying of fruit and seeds releasing after ripening could be counted as necessary part of the plant cycle (Zhang, 2013). Various changes are detectable during over-ripening. By continuous increase in level of cell wall enzymes (see section 1.5.7.2) during ripening and over ripening the rate of softening increased which leads to continues degradation of the cell wall matrix and

consequently result in over softening (Brummell and Harpster, 2001). Also an overall decrease in the activity of antioxidant scavenging system of fruit is detected (Jimenez et al., 2002). The reduction of antioxidant scavenging system and increase of the oxidative stresses are important factors affecting ripening and over-ripening processes in tomato. During over ripening process susceptibility of tomato fruits to pathogens also enhanced (Cantu et al., 2009). During the ripening and over-ripening by dynamics of the cell wall matrix alterations plant cell walls, the first layer of defensive barriers, giving the pathogen this chance to infect the fruit (Cantu et al., 2008a, 2008b). The products from cell wall degradation for instance high level of soluble sugars can also provide nutrients for the pathogen (Jakob et al., 2007; Oeser et al., 2002; Shah et al., 2012). Therefor as the over ripening is a natural process in tomato fruits stages of development and ripening which result in seeds dispersal, preventing the postharvest spoilage is one of the biggest challenges for plant scientists, which means saving millions of dollars and helping the under-nourished population across the world (Klee and Giovannoni, 2011; Seymour et al., 2013b).

## 1.7 Plant breeding methods evolution

### 1.7.1 Classical plant breeding

Identification and selection of desirable traits and their combination to one plant is defined as plant breeding. The first farmers, eight to ten thousand years ago, used plant breeding for obtaining better crops. In 1900, Mendel demonstrated the first scientific basis in plant breeding and the initial bricks of genetic science (Kung, 1993). Manipulation of the combination of chromosomes can be defined as conventional plant breeding, which can be divided in the following categories (Kung, 1993).

#### 1.7.1.1 Selection

The primary method of plant breeding was selection, which means detection of desired trait among population and the process of its breeding to produce a pure line. Selection consists of three main processes. First making a large number of selections from naturally variable original population, followed by growing of each selection progeny for elimination of undesired traits and making pure lines. Finally comparison of selected lines with available commercial varieties (Kung, 1993).

#### 1.7.1.2 Hybridization

Hybridization was the most frequently used method in classical plant improvement methods for gathering the valuable traits in different plants into one line by cross-pollination. The first step was producing pure line for each of traits via self-pollination, followed by outcrossing of the pure lines for yielding progeny with both lines selected traits. The unwanted transferred traits should be omitted with backcrossing, which is still time consuming today. Heterosis is a phenomenon of enhanced vigour of traits, which is obtained by hybridization of highly inbred lines. New hybrid seed should produced every year, as the heterosis effect is just visible in the first generation (Kung, 1993).

#### 1.7.1.3 Polyploidy

Polyploidy means increased number of chromosome sets, which leads to crop improvement. Plants usually have diploid set of chromosomes. The polyploidy occurs spontaneously or by some chemicals (colchicine) could be artificially induced. Enhancing the size and genetic variability is the main effect of polyploidy. But polyploid plants sometimes grow slower and have a lower fertility (Kung, 1993).

#### 1.7.1.4 Induced mutation

Introduction of mutations resulted by chemicals or radiation is another method for finding new improved traits. As the majority of the mutants represent undesirable phenotypic characteristics, this method is no-longer used widely in breeding programs (Kung, 1993).

### 1.7.2 Modern plant breeding strategies

Different techniques have used for modern plant breeding. In this part some important methods are explained and the modern plant breeding methods which were used in this study were explained in general discussion (see sections 6.1.1, 6.1.2 and 6.1.2.3)

#### 1.7.2.1 Restriction Fragment Length Polymorphism (RFLP)

Using RFLP facilitates the conventional plant breeding methods. The faster result of RFLP accompanied with facing smaller populations and decreasing the phenotypical tests and better concluding in plant breeding. In this method the genomic DNA treated with endonucleases restriction enzymes for cutting the DNA in the specific nucleotide sequence. The difference between specious or mutants can be identified based on the differences among DNA fragments number and lengths on the gel with the wild type

lines. These differences have been named as restriction fragment length polymorphism (RFLP). The more similar patterns of bands overlap confer the closer relation between organisms. By looking the banding pattern, it is possible for researchers to identify the lines with specific traits such as resistance genes. Therefore this method facilitates two aspect of: desired trait selection for breeding and also gene identifications (Harms, 1992).

#### 1.7.2.2 Targeting induced local lesions in genomes (TILLING)

Classical chemical induced mutagenesis followed by screening for point mutations. Targeting Induced Local Lesions in Genomes (TILLING) (McCallum et al., 2000) was used for this means as a reverse genetics tool. Mutational screening at a specific locus for detection of the induced lesions was performed in this method (McCallum et al., 2000; Henikoff et al., 2004).TILLING used as a reverse genetic tools for production of GM-free crops, which could alleviate consumer concerns relating to the use of GM foodstuffs. This reverse genetic tool was used successfully in a variety of plant species for instance maize (Till et al., 2004), rice (Till et al., 2007), wheat (Slade et al., 2005) and etc. Also TILLING applied for enhancement of carotenoids in tomato fruits, a 2-fold increase in Lycopene level resulted when TILLING applied on EMS-Red Setter tomato population for lycopene cyclase locus (Minoia et al., 2010; Silletti et al., 2013). In a study trying to apply TILLING on tomato light signal transduction pathway genes, such as *DEETIOLATED1 (DET1), UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1), COP1* and *COP1-like* were characterized. *DET1* represented an increase of 2.3-fold in total carotenoid levels of ripe tomato fruit (Jones et al., 2012). Generally, TILLING provides the facility of production/reproduction of desired mutated genes via a non-GM method.

#### 1.7.2.3 System biology

Massive datasets can be obtained from “omic” data, for instance proteomics, metabolomics, genomics, transcriptomics and also the more recent data from Microarrays and RNA-Seq data. It has become a priority to develop computational tools for integrating and analysing them efficiently (Yuan et al., 2008; Wang et al., 2009b). These sets of data hold many hidden useful information for study of plant chemical diversity and also gene functions in different biological processes, for instance developmental programs and stress responses (Oksman-Caldentey and Saito, 2005). In the recent years for this means some web-based systems, which analysing

the large scale gene expression data in correlation with metabolite profile data sets and other omics data along with consideration of diverse biological information, have been developed (Joung et al., 2009). It is hoped that system biology could enhance and maximise revealing the information from the existed data.

#### 1.7.2.4 Revolution in future of gene manipulation: Specific DNA editing by cutting-edge CRISPR technology

Clustered regularly-interspaced short palindromic repeats (CRISPR) are repeated segments of prokaryotic DNA which followed by a spacer DNA (from previously exposed virus of plasmid) (Marraffini and Sontheimer, 2010). CRISPR system provide an immune system for prokaryotes against plasmids and phage (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). This system by recognition and omission of foreign DNA protect the cell. This mechanism is similar to RNA interference in eukaryotes (Marraffini and Sontheimer, 2010). CRISPR technology domesticated for targeted genome editing via engineering of some nucleases such as Cas9. This new approach results in generating RNA-guided nucleases (Cas9) with customizable specificities (Sander and Joung, 2014). By transferring the Cas9 protein and the relevant guide RNAs into a cell, the organism's genome can be edited at any desired location (Sander and Joung, 2014; Ledford, 2015; Hendel et al., 2015). The schematic mechanism of CRISPR is illustrated in Figure 1-13. This new method could solve many problem in high value compound improvement in plant science as do not have the negative points of gene manipulation (Mali et al., 2013).

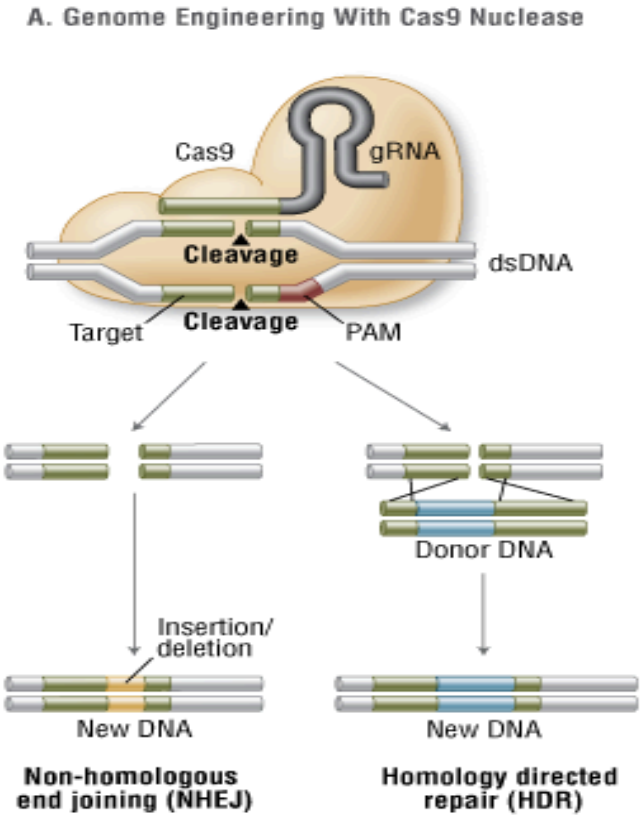



Figure 1-13 CRISPR/CAS9 genome engineering (adapted from Biolabs, 2007).

The guide RNA (gRNA) is joined with the DNA sequence, which finds the host gene of interest. The gRNA targets specific sequences on the host RNA and make a complex with Cas9 that join the protospacer adjacent motif (PAM) on the target and apply its endonuclease function to create double stranded breaks. This could result in two mechanisms for repair. First method is non-homologous end-joining (NHEJ), which result in mutations in the double stranded breaks site. Homologous recombination (HR) is the other mechanism, which enables the new introduced DNA information to be placed at the break site.

## 1.8 Aims and objectives

This study is consisted of two aims:

The first aim is the evaluation of different strategies for the enhancement in the production of high-value carotenoids and ketocarotenoids in tomato fruit. This aim followed through the following objectives:

- In the first objective the strategy used for enhancement of carotenoids was identification and validation of putative transcription factors and genes affecting ripening and carotenoids accumulation; which were consisted of three parts
  A. Identification of the putative homologue transcription factor of *APRR2-Like* on chromosome 6. *APRR2* transcription factor [ARABIDOPSIS PSEUDO-RESPONSE REGULATOR-2] is an important TF affecting fruit development and ripening (Fitter et al., 2002). In tomato *APRR2-Like* is shown that affect the accumulation of pigments in fruit. *APRR2-Like* TF in tomato genome assembly (The Tomato Genome Consortium) was designated as Solyc08g077230 (Pan et al., 2013). *APRR2-Like* is located on chromosome 8 and we search for its homologue gene on chromosome 6 with the same probable effects on pigmentation.
  B. Characterisation of Solyc06g053960 TF designated as *ESB9*. Some transgenic lines (ESB lines) were created in result of RHUL and Syngenta Company collaborations. These TFs are candidate genes for some tomato ripening regulators. These stable transgenic lines are downstream TFs derived from the master regulator *RIN*, which are candidate genes potentially effecting fruit colour, flavour and texture. In this part the primary characterisation of 10 independent transgenic ESB9 lines were carried out in the first generation.
  C. Putative candidate genes/regions in the *S.pennelli* sub ILs of 2-4 and 2-5 (Q1968 line) were studied also under this strategy. Systems Biology and QTL mapping has identified a number of regulatory genes associated with fruit pigmentation. The SubIL of 2-3/2-4 from *S.pennellii,* designated Q1968 was found to have high lycopene content compared to the wild type counterpart (Seymour et al., unpublished). This region underlying two

putative TFs. Different strategies like metabolomics and transcriptomics have been performed to investigate the potential effect of these two TFs on carotenoid production especially lycopene.

- In the second objective for the enhancement of high-value carotenoids and ketocarotenoids in tomato fruit the genetic cross of between putative lines were undertaken:
  A. The first cross made between regulatory gene of *CrtZW* (transgene encodes the *β-carotene ketolase/hydroxylase* gene which introduce new pigments of ketocarotenoids to tomato) (Enfissi et al., unpublished) and transcriptional regulator of *DET1 (DE-ETIOLATED1* down-regulated line which considerably enhanced carotenoids content) (Enfissi et al., 2010; Davuluri et al., 2005). The new genotypes of CrtZW.DET1 were characterised for ketocarotenoids and carotenoids improvements.
  B. The second cross made between regulatory gene of *CrtZW* and transcriptional regulator of *APRR2-Like* gene (which the transgene line indicated enhanced chlorophylls and carotenoids levels). The new genotypes of CrtZW.APRR2-Like were also characterised for ketocarotenoids content and carotenoids improvements.

The second aim is understanding the mechanisms underlying the extended shelf life observed in the varieties of CrtZW (transgene encodes the *β-carotene ketolase/hydroxylase* gene) and CrtZW.DET1 (CrtZW line were crossed with the *DE-ETIOLATED1* (*DET1*) down-regulated line) of *solanum lycopersicum* fruit.

- To achieve this aim the following objective was followed:Third objective focused on extended shelf life and late ripening property of CrtZW.DET1. This genotype displayed delayed ripening, leading to the hypothesis that this delay occurs via modulation of abscisic acid (ABA) content in the fruit due to the depletion of β ring derived carotenoids. Associated changes in cell-wall degradation enzyme activities, hormones level and cell wall compound measurements were also evaluated. These experiments were directed towards elucidating a new mechanism for improving the shelf life characteristics of fleshy fruits.

# CHAPTER 2: MATERIAL AND METHODS

## 2.1 Plant cultivation, phenotyping and collection

### 2.1.1 Tomato cultivation

All tomato plants were sown in pots containing M3 growing medium (Scotts Levington®, UK). The size of pots was changed two times, as the plants were cultivated to maturity.

All plants were grown under greenhouse conditions with supplementary lighting. A daytime temperature of 20 to 25°C, with a night time temperature of 15°C, with 8 hours of darkness were used. Tomato fruits were tagged at breaker stage for the different stages of ripening collection. The stages of ripening were considered as Mature Green (MG), Breaker (Br), 3 days post breaker (3dpb), 7 days post breaker (7dpb), 14 days after breaker (14dpb) and Storage (S) stages. For more detailed explanation see section 2.1.3.2. For each stages of ripening three technical samples (three fruits) from each of three independent biological samples (three different plant) were harvested (total of 9 fruit for each stages of ripening) and analysed or stored as explained in section 2.1.3. This sampling was applied for all of the analysed genotypes unless otherwise stated.

### 2.1.2 Phenotype evaluation

All plants were investigated for phenotypic differences. These parameters included morphological differences such as, leaf colour, fruits colour, truss numbers, fruits numbers, firmness and weight. Differences in visual phenotypes were photographed using a Sony digital camera.

"Firmness" in food materials is similar to "stiffness" in engineering materials which is described by the force-deformation relationship or amount of tester probe penetration. Firmness is usually used to evaluate fruit quality and it is directly related to stages of fruit development, ripening and storage potential; different ways used to estimate firmness for instance manual touch or use of penetration tests which include destructive (damage the fruit therefore the sample is discarded) and non-destructive (no damage to fruit and the experiments can be continued) methods (Sobotka et al., 1972; Lesage and Destain, 1996).

In this study a non-destructive method was used. Firmness factor of fruit was measured with a firmness meter Qualitest™ with 0.25cm$^2$ probe. The principle of the method is based on the millimetre of probe penetration into the fruit peel. This amount is presented as the percentage of Fruit firmness factor (Fff) in the indicator

screen. Therefore, the data is shown between 0 in over ripe tomatoes to 100 in immature green tomatoes and the unit is percentage of Fff.

### 2.1.3 Tissue collection

#### 2.1.3.1 Tissue collection for DNA/RNA analysis

Leaf samples were chosen from young growing plants. Fruit tissues were harvested at predefined stages of ripening. At least three to four leaves or fruits were collected from each biological replicate at each stage and pooled prior to experiment. These constituted one biological replicate. All experiments were done with a minimum of three biological replicates unless otherwise stated. Pericarp tissues of tomatoes were deseeded, chopped and rapidly frozen in liquid nitrogen. Samples were stored at -80°C prior to Nucleic acid experiments.

#### 2.1.3.2 Defining the stages of ripening

Fruits were harvested in different stages of development/ripening for different analysis. Tomato varieties with normal pattern of ripening stages (T56, Money Maker, DET1, AC, ESB9, Microtom and the lines derived from their crosses) were harvested in Mature Green (MG) when the fruit is in its full size and green. Breaker (Br) defined as the time first sign of colour change (a spot of yellow) appear in fruit. The approximate hour of breaker time was considered for counting the stages of fruit ripening and sampling mostly happened between 10 am to 3pm. 3dpb is 3 days post breaker and 7dpb is 7 days post breaker which fruits are ripe at this stage. 14dpb refer to fruits that stay on plant for 14 days after breaker. Storage (S) consist of 7 days storage of +7 fruits in 10°C and darkness.

For tomato varieties with delay in ripening (CrtZW, CrtZW.DET, Mic CrtZW, Mic CrtZW.APRR2) the definition of stages of ripening were changed as they did not follow the normal 7 dpb ripening pattern of wild type varieties. The definitions represent in section 4.2.1.3.

#### 2.1.3.3 Tissue collection for metabolite analysis

Samples for metabolite analysis were harvested and chopped as stated in section 2.1.3.1. Tissues were stored at -80°C and when samples from all stages of ripening were obtained freeze drying was performed for 3 days, until the samples were completely dry (e.g. weight was constant) and stored at -20°C. All metabolite

analyses were performed over a maximum one month after freeze drying to avoid unintended metabolite changes in samples.

### 2.1.4 Seed collection

Seeds were collected from fully ripe fruits and treated with (1:1 HCl (32%) /dH2O) for 30 min to one hour then washed and air dried before storage in paper envelope.

## 2.2 Extraction and analysis of metabolites

### 2.2.1 Sample preparation and extraction of metabolites

#### 2.2.1.1 Pigment extraction for UPLC analysis

Freeze dried fruits and leaf tissues were used for extraction of pigments and tocopherols. Collected freeze dried tissues (as detailed in section 2.1.3.3) were ground using a TissueLyser LT (Qiagen, UK) in 2 ml screw-cap tubes with a 0.5 mm stainless steel bead (Qiagen, UK). Homogenised material (10mg) was weighed in clean microcentrifuge tubes (1.5 ml) as three technical replicates. Methanol (250µl) was added and mixed vigorously with a vortex then chloroform (500µl) added and mixed. Tubes were stored in ice for 20 min for better extraction of pigments. Subsequently, water (250µl) was added. Samples were centrifuged at 14,000 rpm in an Eppendorf 5810R centrifuge for 5 min. The non-polar phase containing pigments was transferred to a new microcentrifuge tube (1.5 ml). The aqueous phase was re-extracted with chloroform (500µl). Non-polar organic phases were pooled together and dried using a GeneVac Ez-2 Plus rotary evaporator (UK). Dried samples were stored at -20°C prior to analysis.

#### 2.2.1.2 Metabolite extraction for GC-MS analysis

Freeze dried fruit tissues were extracted for the analysis of polar phase metabolites. Three biological replicates each with a minimum of three technical replicates were used. Methanol/water (800µl; 1:1, v/v) was added to the 2 ml microcentrifuge tube containing freeze-dried grounded tissue (10 mg). After mixing vigorously with a mixer, the sample was stirred at room temperature for 1 hour. Chloroform (800µl) was added and mixed. Samples were then centrifuged at 10,000 rpm for 5 min. The upper phase containing methanol and water transferred to a new microcentrifuge tube. Non-polar phase (20µl) was transferred to new screw-cap glass vial and 10 µg of ribitol internal standard (ribitol was chosen based on its similarity to at least one of

compounds in samples but with a completely distinct peak from all of analysts; because any factor which affect samples will also affect the internal standard peak with the same degree) spiked in sample prior to drying using a GeneVac Ez-2 Plus rotary evaporator (UK). At this stage samples could whether be stored at -20°C or stepped to derivatisation.

#### 2.2.1.3 Metabolites derivatisation for GC-MS analysis

Derivatisation was performed on prepared sample as described in section 2.2.1.2. Methoxyamine-HCl (Sigma-Aldrich, UK) was prepared at a concentration of 20 mg/ml in pyridine and 30µl was added to sample. After 1 hour storage at 40°C, N-methyltrimethylsilytrifluoroacetaminde (70µl; Sigma-Aldrich, UK) was added to sample and incubated for 2 hours at 40°C. Sample was used for GC-MS analysis (section 2.2.4). The maximum storage time for derivatised sample was 24 hour at room temperature before running on GC-MS.

### 2.2.2 Spectrophotometric analysis

#### 2.2.2.1 Colorimetric assays (enzyme assays and uronic acid measurement)

A Spectrophotometer was used for colorimetric assays. The absorbance of desired wavelength was read for each sample with three replicates using a DU®800 Spectrophotometer (Beckman Coulter, UK). The absorbance of enzyme assays and uronic acid measurement were used in a specific calculation for final results. (See sections 2.7.2, 2.8.2 and 2.8.3)

#### 2.2.2.2 Pigment quantification and standard curve

The extinction coefficient of most pigments (1% w/v sol) was measured in a 1 cm path-width cuvette and have been published by (Britton G et al., 1995). Below equation can be used for weight quantification (X; mg) of carotenoids by using a simple spectrophotometer to measure the absorbance (Abs), while Y is the sample solution (ml).

$X = (Abs \times Y \times 1000)/(absorbance\ coefficient \times 100)$

Quantification of carotenoids was calculated by dissolving the desired carotenoid in the appropriate solvent. Absorbance of samples were measured at the maximum wavelength for each carotenoid in the specified solvent using a scanning DU®800 Spectrophotometer (Beckman Coulter, UK). A dose response curve was created for

each compound by plotting different known concentrations of compound against the obtained area of spectrum in UPLC. The spectra of desired metabolites illustrated in Appendix1 Figure A 7-1. A sample of standard curve and the equations of carotenoids, ketocarotenoids and α-tocopherol are presented in Appendix 2 Figure A 8-1.

### 2.2.3 Ultra high performance liquid chromatography and data analysis

Analysis of pigments and tocopherols was performed using an Acquity™ system Ultra High Performance Liquid Chromatography (Waters, UK). The separation were carried out on a reverse phase BEH column (2.1 x 100 mm, C18, 1.7 μm) and coupled with a BEH VanGuard pre-column (2.1 x 50 mm, C18, 1.7 μm). The column and samples were maintained at 30°C and 8°C respectively.

The continuous on-line scan mode of the UV/VIS was detected from 200-600 nm using an extended wavelength PDA (photo diode array) (Waters, UK). Each pigment was identified by its characteristic spectrum at its maximum wavelength. Carotenoids were quantified by counting the dose-response area of each pigment. The used mobile phases were A, MeOH/$H_2O$ (1:1, v/v) and B, ACN (acetonitrile)/ethyl acetate (3:1, v/v). HPLC grade solvents from Fisher Sientific were used and filtered prior to use.

Two different methods were used forcarotenoids and ketocarotenoids. For carotenoids, the gradients used were 30% A: 70% B for 0.5 min, then 0.1% A: 99.9% B for 5.5 min and finally to 30% A: 70% B for the last 2 min. For ketocarotenoids, the gradients of solvents were 50% A: 50% B for 0.5 min and then 30% A: 70% B for 4.5 min and stepped to 0% A: 100% B for 2 min, back to 30% A: 70% B for 1 min. The final isocratic step was 50% A: 50% B for the two last minutes in order to return the column to its initial conditions.

Identification of carotenoids was performed by the comparison of spectral characteristics and chromatographic properties. Dose response curves for each carotenoid pigment was created by running different concentrations of authentic standard on the UPLC. Carotenoids were monitored at wavelengths of 450 nm (colored carotenoids), 350 nm (phytofluene) and 286 nm (alpha- tocopherol and phytoene), which were selected from a continuous monitoring mode across 250 to 660 nm. Peak areas corresponding to individual carotenoids were used to construct the dose response curve.

### 2.2.4 Gas chromatography–mass spectrometry and data analysis

Gas Chromatography-Mass Spectrometry analysis was performed to analysis of metabolites in polar phase. GC analysis was carried out on an Agilent HP6890 (UK) gas chromatograph with a 5973MSD as described by (Enfissi et al., 2010).

The carrier gas was Helium with a flow rate of 0.5 ml/min. The samples (1µl) were injected splitless (by an autosampler) through the inlet (280°C) into a DB-5MS column (30m x 250 µm x 0.25 µm) (J&W Scientific). The separation temperature gradient was 4 min at 70°C then ramping at 5°C/min to 320°C. Further 10 min holding at 320°C was carried out, creating total time of 60 min. Temperature of transfer was set at 250°C before samples entered to MS detector. MS was performed in full scan mode with 70eV positive electron ionisation (EI), which scanned from m/z[1] 10 to 800. A specific mass spectral (MS) library was established from in-house standards created and developed by Dr. Laura Perez in the lab for known tomato metabolites. For identification of each metabolite, retention indices and MS were compared to customised libraries. Retention time calibration was performed on all of standards to facilitate identification of retention indices (RIs).

Relative quantification to an internal standard was used by AMDIS (version 2.69 (2010)). The generated reports with AMDIS were transferred to Excel (Microsoft Office 2007) to normalise the data towards internal standard (ribitol) (see section 2.2.1.2).

The mean ratios ± standard deviation (S.D.) was calculated and compared to the control. Normalised data was analysed using PCA (principal component analysis) in SIMCA Pv13 (Umetrics AB) and Anova (when 2 set of samples were compared) or Student Dunnett's test (when more than 2 set of samples were compared) based on experiment was performed. Statistical significant changes ($P<0.05$) of metabolites were overlaid to a pre-created in-house pathway diagrams with BioSynLab© software.

## 2.3 DNA and RNA molecular analysis

### 2.3.1 DNA and RNA extraction

#### 2.3.1.1 DNA extraction

DNA was extracted from leaf and fruit tissue using Qiagen DNAeasy® Plant Mini Kit.

[1] The "m" indicates the molecular or atomic mass number and "z" the charge number of the ion.

All steps were performed as described in the manufacture's instruction. Collected frozen tissue (section 2.1.3.1) was grounded using a mortar and pestle. All steps were performed on dry ice and continuously small amount of liquid nitrogen were added to samples to prevent thawing during their grinding. Samples were kept on dry ice before extraction. Grounded tissues (60-90 mg) were weighted into new pre chilled 1.5 ml sterile microcentrifuge tubes. Buffer AP1 (400µl) with RNase A (4µl) were added to samples then mixed vigorously by a vortex. The tubes were stored for 10 min at 65°C. Samples mixed three times during the incubation. Subsequently, buffer AP2 (130µl) was added and mixed. Incubation time for this step was 5 min on ice. The lysate was transferred into a QIAshredder Mini spin column coupled with a 2 ml collection tube and centrifuged at 14,000 rpm for 2 min with an Eppendorf 5810R centrifuge. After transferring the flow-through to a new sterile microcentrifuge tube, buffer AP3 (1.5 volumes) was added. The sample was transferred to a Dneasy Mini spin column then centrifuged at 14,000 rpm for 1 min. The column was transferred to a new 2 ml collection tube and washed twice with buffer AW (500µl). The column was centrifuged for 1 min at 14,000 rpm for each wash. The column was transferred to a sterile microcentrifuge tube (1.5 ml). DNA was eluted with buffer AE (50µl) and centrifuged at 14,000 rpm for 1 min. Extracted DNA was stored at -20°C.

#### 2.3.1.2 RNA extraction

RNA extraction was initiated from frozen grounded samples (as detailed in sections 2.1.3.1 and 2.3.1.1) using the Qiagen RNeasy® Mini Kit. RNase-Free DNase set (Qiagen, UK) was used as a complementary step for deleting of cellular DNA.

Frozen grounded tissue (three replicates; up to 100 mg) was transferred to a pre-chilled sterile microcentrifuge tube (1.5 ml). Buffer RLT (450µl) was added to the tubes and mixed vigorously with a vortex. Samples were incubate (1-3 min) at 56°C. The lysate was transferred into a QIAshredder Mini spin column coupled with a collection tube (2 ml). The column was centrifuged at maximum speed with an §Eppendorf 5810R centrifuge for 2 min. The flow-through was placed into a fresh sterile microcentrifuge tube. Next, 100% ethanol (0.5 by vol.) was added. The lysate was transferred in an RNeasy Mini spin column with a 2 ml collection tube. Sample was centrifuged for 15 sec at 14,000 rpm and flow-through discarded. Buffer RW1 (350µl) was added. The column was centrifuged for 15 sec at 14,000 rpm and the

flow-through once again discarded. In this step, the complementary DNA removal was applied. DNaseI mixture was prepared by mixing RDD buffer and DNaseI (7:1, v/v). DNaseI (80µl) was applied directly to the membrane of RNeasy column. An incubation of 15 min at room temperature was applied. Then three steps of washing were applied. Buffer RW1 (350µl) was added for first wash of the membrane, followed by another wash with buffer RPE (500µl). Then the column was centrifuged at 14,000 rpm for 15 sec for each wash. Final wash was performed with buffer RPE (500µl) and the column centrifuged for 1min at 14,000 rpm. The collection tube was changed and another 1min centrifugation applied. The column was transferred into a new sterile microcentrifuge tube. The RNA was eluted with RNase-free water (40µl) by centrifugation at 14,000 rpm for 1 min. RNA solution was diluted to desired concentration and aliquoted prior to storage at -80°C.

#### 2.3.1.3 Quantification and qualification of nucleic acids

Nucleic acid quantification was performed using a NanoDrop 1000 spectrophotometer v3.7. This Spectrophotometer used low volume of sample (1µl) and scanned the sample between 220 to 360 nm. In the primary setting, the main chosen category was Nucleic Acid and the sub category for DNA and RNA was DNA-50 and RNA-40 respectively. Before starting, the NanoDrop apparatus was blanked with sterile deionised water. Different absorbance ratios were displayed on screen by automated ratio calculation. These ratios were used as an indication of the relative purity of the DNA and RNA samples. The accepted values for 260/230 ratio and 260/280 ratio was ~2. Lower ratios that indicated contaminations were not accepted. Nano drop also provided the concentration of samples (ng/µl), which was used for making the desired aliquots.

Detection limits based on manufacture guidance for double strand DNA, single strand DNA and RNA was maximum of 3700, 2400 and 3000 ng/µl respectively and minimum of 2 ng/µl.

Agarose gel electrophoresis was performed (see section 2.3.2.3) for testing the integrity of extracted RNA samples (Section 2.3.1.2). RNA (300-500 ng) was loaded to wells for testing the integrity of RNA. The accepted Integrity of RNA was the approximate 2:1 intensity of 28S rRNA to 18S rRNA which approximately were accepted by observation.

For RNA-Seq, extracted RNAs after quantification and qualification were pooled

together and Biomatrica® RNAstabilizer (20µl) was added to each samples. Following the manufacture guidance, samples were dried using a GeneVac Ez-2 Plus rotatory evaporator (UK) and stored in room temperature.

### 2.3.2 Molecular methods to detect the transgene

#### 2.3.2.1 Primer designing

Primers for PCR and real-time PCR were designed using an open-source program Primer3web version 4.0.0 (http://primer3.ut.ee/). Sequence of the genes of interest was obtained from Sol Genomic Network (http://solgenomics.net/search/loci). PCR primers was designed for the length of 18-23bp (optimal 20bp), GC content of 30-70% (optimal 50%), Tm of 57-62°C (optimal 59°C) with less than 5°C difference. For real-time PCR primers, the amplicon length was designed between 100-150bp with the primer length of 10-30bp. The GC content ideally was chosen between 40-60% and Tm: 55-60°C with the maximum difference of 4°C. Primers were made by Eurofins MWG (Operon, UK) (see Table 2-1).

Table 2-1 Sequences of primers used in real-time PCR and PCR.

DXS, 1-deoxy-D-xylulose 5-phosphate synthase; PSY1, phytoene synthase-1; PSY2, phytoene synthase 2; PDS, phytoene desaturase; LCYB, β- lycopene cyclase; CYCB, β- lycopene cyclase; CrtZ, carotene hydroxylase; CrtW, carotene ketolase; DET1, de-etiolated1 gene; APRR2-Like, Arabidopsis pseudo response regulator2-Like gene; Solyc 02g082040, transcription factor MYB86-Like; Solyc 02g081780, B3 domain-containing protein transcriptional factor; NptII, kanamycin resistance gene.

| Gene ID | Accession No | Forward sequence | Reverse sequence |
|---|---|---|---|
| *DXS* | AF143812 | GCGGAGCTATTTCACATGGT | CTGCTGAGCATCCCAAT |
| *PSY*1 | Y00521 | TGGCCCAAACGCATCATATA | CACCATCGAGCATGTCAAATG |
| *PSY*2 | L23424 | GATGATGGCCCTAATGCATCA | TCAAGCATATCAAATGGCCG |
| *LCY*B | AF254793 | TCGTTGGAATCGGTGGTACAG | AGCTAGTGT CCTTGCCACCAT |
| *CYC*B | Y18297 | TGTTATTGAGGAAGAGAAATGTGTGAT | TCCCACCAATAGCCATAACATTTT |
| *Actin* | BT013524 | AGGTATTGTGTTGGACTCTGGTGA | ACGGAGAATGGCATGTGGAA |
| *CrtZ* | AB181388.1 | TGGGAATGGAGGCTTTCGCTTGG | ACGATAGCAGGAGCAGCGAAG |
| *CrtW* | AB181388.1 | TCTGGGCTGCTCCTGCTCTTC | AGCGTGAGCATCAGCGA |
| *DET1* | AJ224356 | TTGACGAAAGGGTTTTCCAC | GAATTCCCCAATTGCTCGTA |
| *APRR2-Like* | KC147634 | TTGCCCCAGGTGGTCAGTTCTAT | CAGTGCCAAGTCTCTGCAGGTTT |
| Solyc 02g082040 | XM_004232564 | AAACACATCAAGACCCATGG | CCCCAAAATTCTGTGTACGT |
| Solyc 02g081780 | XM_004233720 | TGTTGACGAAGGCTTGTTTAC | AAACAGCAAAACATCGCCTT |
| PCR primer | | | |
| *NptII* | | CAGGTTCTCCGGCCGCTTGG | CTGCGCTGACAGCCGGAACA |

#### 2.3.2.2 PCR

PCR was performed for detection of existence of ESB9 (Solyc06g053960) gene in 9 line of transformed plant (UC Davis facility (USA)) see section 3.2.2.1. PCR reaction was operated using a Techgene thermo cycler (Techne, UK) and Illustra™ puReTaq Ready-to-go PCR beads (GE Healthcare, UK). For each reaction, sterile high-quality water (Sigma-Aldrich, UK) (22µl) was added to tube. DNA template (around 50 ng for genomic DNA) and forward and reverse primers (10 pmol each) were added to tubes. Usually the desired amounts of template DNA and primers were obtained in 1µl each for a final volume of 25µl. In case that this volume changed, the volume of water changed for a final volume of 25. The program of thermo cycle was arranged for 30 cycles of amplification with a pre-denaturing step at 95°C for 5 min. PCR amplification program included the following steps: denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec and a final incubation of 5 min at 72°C for completion of reaction.

#### 2.3.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for checking the integrity of extracted RNA, size of fragment and existence of PCR product. Depend on the size of PCR product, the concentration of agarose gel (usually ~1% (w/v)) was chosen. Agarose powder was dissolved in TBE (Tris- hydroxymethyl-aminomethane (Tris) borate ethylenediamine tetra-acetic acid) by heating in a microwave. A nucleic acid gel stain (GelRed™; Biotium, UK) was added prior to pouring in a gel set container with appropriate comb and left for 20 min to set. The gel was taken out from the container and was cleaned in all sides before placing in an electrophoresis tank, which was filled with TBE. Samples were mixed with Blue/Orange Loading Dye (6X; Promega, UK) and were loaded in gel wells. Depending on the sample kind (DNA or RNA) and their size, appropriate ladder was chosen (100bp or 1kb; Promega) and run for the size comparison alongside samples. The gel was run for 20 min at 100 volts for a fast run or a slow run of 45 min at 50 volts. Visualization of DNA bands were done by using a UV Genius3 gel imaging system (Syngene, UK).

### 2.3.3 Transcript level quantification

#### 2.3.3.1 Standard preparation for RT-qPCR

##### 2.3.3.1.1 RT-PCR

Reverse-transcription PCR (RT-PCR) was performed for the production of complementary DNAs (cDNA) corresponding to genes of interest using illustra™ Ready-to-go™ RT-PCR beads (GE Healthcare, UK). Extracted RNA (described in section 2.3.1.2) was used for RT-PCR. Double distilled sterile water was added to illustra™ Ready-to-go™ RT-PCR beads (GE Healthcare, UK) and stored in ice until dissolved. Then oligo (dT) primer (2.5 pmol) and RNA template (~200 ng) was added. After adding all components, tubes were mixed well by flicking and spun for some seconds before incubation in a Techgene thermo cycler (Techne, UK). At the first step, tubes were incubated for 5 min at 55°C, followed by 30 min incubation at 42°C for reverse transcription reaction and a final thermal step of 5 min at 95°C for denaturing of reverse transcriptase. After this step, forward and reverse primers of specific gene were added to tubes, mixed and followed by 30 cycles of PCR amplification as follows: denaturation at 94°C for 30 sec; annealing at 48°C for 30 sec; extension 72°C for 30 sec and finally, incubation of 5 min at 72°C. The accuracy of PCR reaction was checked with agarose gel electrophoresis (section 2.3.2.3). If needed, the sample was purified by Wizard® SV Gel and PCR Clean-Up System (Promega, UK) (detailed in section 2.3.3.1.2). Finally the desired gene was cloned in a TOPO® vectors (section 2.3.3.1.4) for real-time RT-qPCR.

##### 2.3.3.1.2 DNA purification

DNA purification was used for RT-PCR and PCR amplification. In RT-PCR, the DNA was purified for a better efficiency before cloning of cDNA in TOPO® vectors. Wizard® SV Gel and PCR Clean-Up System (Promega, UK) were used following the manufacture's protocol. Membrane Binding Solution (equal to the volume of PCR product) was added to tube, mixed by vortexing, transferred to a SV Minicolumn with a collection tube and incubated at room temperature for 1 min. Samples were centrifuged at 14,000 rpm for 1 min using an Eppendorf 5810R centrifuge. The flow-through was discarded and Minicolumn was reinserted into Collection Tube.

Two steps of washing were performed using the Membrane Wash Solution (700µl and 500µl), followed by 1 min and 5 min centrifugation at 14,000 rpm respectively.

The column was re-centrifuged for 1 minute at 14,000 rpm with the lid of the microcentrifuge remaining open to allow evaporation of any residual ethanol. The mini column was placed in a new sterile 1.5 ml microcentrifuge tube, Nuclease-Free Water (50µl) was added to center of column and incubated for 1 min at room temperature. DNA was eluted by centrifugation at 14,000 rpm for 1 min and stored at -20°C. The purified DNA was used for cloning into TOPO® vector (section 2.3.3.1.4) for use as real-time RT-qPCR standard.

#### 2.3.3.1.3 Media preparation and bacterial cultures

*Escherichia coli* strain DH5™ –T1$^{R}$ was used for plasmid amplification. The media Luria Broth (LB) was used for bacterial growth. Luria Broth contained tryptone (1% (w/v)), yeast extract (0.5 % (w/v)) and NaCl (1% (w/v)). Agar (1.5% w/v) was added to create solid LB media. Media were autoclaved for 20 min at 121°C. Appropriate antibiotic was prepared, filtered and added separately to falcon tubes containing LB and LB agar. The LB media supplemented with agar was transferred to Petri dishes and allowed to set on a level surface at room temp within a laminar flow hood. LB agar plates were incubated at 37°C overnight for the growth of bacterial colonies. Liquid LB cultures were shaken at 180 rpm at 37°C for 12 h.

Glycerol stocks were prepared from liquid cultures by pipetting of culture (750µl) to Glycerol (100%; 250µl) in pre-autoclaved 2 ml screw-cap tubes. The stock was mixed well by pipetting and placed at -80°C for long-term storage.

For re-culturing of frozen bacterial cells, some media containing cells was scraped from the surface of the frozen glycerol stock under sterile conditions and streaked to LB agar plates containing the antibiotic and incubated at 37°C for growth of the bacterial colonies.

#### 2.3.3.1.4 DNA cloning in TOPO® vector

RT-PCR products were cloned in to pCR®2.1 TOPO® cloning vectors using the TOPO-TA DNA cloning® kit (Invitrogen, UK). Cloning mixture contained cDNA (2µl), salt solution (1µl) and water to a final volume of 6µl. An incubation of 5 min at room temperature was performed for the ligation of cDNA into the vector. The tube containing the mixture described was placed on ice. One Shot®TOP10 chemically competent *E.coli*, where thawed on ice prior to use, and transformed by a heat shock at 42°C for 30 sec then immediately stored on ice. S.O.C medium (room temperature; 250µl) was added to tube and then shaken at 200 rpm for 1 h at 37°C using a shaking

incubator. Pre-warmed LB agar plates containing kanamycin (50 µg/ml) for colony selection were prepared. The transformation reaction (50-150µl) was streaked onto plates and incubated overnight at 37°C. A single colony was transferred to new LB liquid (5ml) culture with supplemented kanamycin (50 µg/ml) and incubated overnight in a shaking incubator at 37°C. Samples were purified by Wizard® *Plus* SV Miniprep DNA Purification System (detailed in section 2.3.3.1.5).

#### 2.3.3.1.5 Plasmid DNA purification from bacterial culture

Wizard® *Plus* SV Minipreps DNA Purification System (Promega, UK) was used for purification of plasmid DNA following the manufacture's protocol. LB media (5 ml) containing an overnight bacterial culture of desired amplicon of gene with specific antibiotic selection within vector (section 2.3.3.1.4) was centrifuged and pelleted at 4,000 rpm for 5 min using a HERAEUS Pico17 centrifuge. The liquid phase was discarded and the pellet dissolved in Cell Re-suspension Solution (250µl) and placed into a fresh 1.5 ml microcentrifuge tube. Cell Lysis Solution (250µl) was added and inverted four times for mixing. Alkaline Protease Solution (10µl) was added and inverted for a further four times, then incubated at room temperature for 5 min. Neutralization Solution (350µl) was added and mixed by inverting (four times). The sample was centrifuged for 10 min at 14,000 using an Eppendorf centrifuge 5810R. The cleared lysate was transferred to a Spin column coupled with a 2 ml collection tube and centrifuged for 1 min at 14,000 rpm. The flow-through was discarded. The column was washed two times with Wash Solution (750µl and 250µl) and centrifuged for 2 min at 14,000 rpm. The column was placed in a clean 1.5 ml microcentrifuge and Nuclease-Free Water (100µl) added for elution of DNA performed by centrifugation at 14,000 rpm for 1 min. The quality and quantity of samples were checked using a Thermo Scientific NanoDrop™ 1000 and agarose gel electrophoresis (sections 2.3.1.3 and 2.3.2.3). This DNA was used as the standard template in RT-qPCR experiment. DNA solutions were stored at -20°C.

### 2.3.3.2 RT-qPCR

For relative expression quantification of gene of interest Real-time qRT-PCR was performed using a QuantiFast™ SYBR® Green RT-PCR kit (Qiagen, UK) with a thermal cycler of Research Rotor-Gene RG-3000 (Corbett Life Sciences, UK). Extracted RNA (as described in section 2.3.1.2) was aliquoted to 25 ng/µl and stored

at -80 prior to usage. A master mix was prepared for each gene separately which consisted of 2X QuantiFast SYBR Green RT-PCR Master Mix (10µl for 1 well; multiplied by the number of wells needed plus 1), sterile RNA-free water to a final volume of 20µl for 1 well (multiplied by the number of wells needed plus 1), forward and reverse primers (1 µM for 1 well; multiplied by the number of wells needed plus 1) and at the end QuantiFast RT Mix (0.2µl per reaction; multiplied by the number of wells needed plus 1). The master mix was mixed by inverting for some times, spun down and stored on ice before aliquoting into wells. This step was carried out as quickly as possible due to sensitivity of QuantiFast RT Mix. Then amplicon DNA and RNA template were added for standard curve construction and the determination of the transcript levels for the gene of interest respectively. Triplicates of each reaction were performed. The lids were placed and subsequently qPCR tubes placed in Research Rotor-Gene RG-3000. The Real-Time Cycler conditions used were a 10 min incubation at 50°C for reverse transcription followed by 5 min incubation at 95°C for activation of HotStarTaq DNA polymerase and at least 30 cycles of 10 sec at 95°C and 30 sec at 60°C. Melt curve analysis was performed for understanding of the specificity of the reactions. The calibration curve was created with every set of run for each gene of interest, using at least 4 to 5 different concentration of prepared serial dilution a of standard (RT-qPCR standard produced as detailed in section 2.3.3.1). An amplicon of the Actin gene was used for normalisation, as its expression was similar throughout all growth stages. The acceptable efficiency for reaction was between 0.9 and 1.1 and the $R^2$ value (correlation coefficient shows how the data points lie on line) of more than 0.985. The methods used to analyse data is detailed in section 2.3.3.3.

#### 2.3.3.3 RT-qPCR analysis

Relative quantification of gene expression was performed by comparing *Ct* values (cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold values), these parameters were measured using a Rotor-Gene Analysis software 6.1. The acceptable efficiency for both of calibrator gene (actin gene) and the gene of interest were between 0.9 and 1.1 with a maximum difference of 10%. The standard curve indicated the reaction efficiency, sensitivity, linearity and reproducibility of the assay. Relative quantification was followed by below equation:

$\Delta\Delta Ct = \Delta Ct$ calibrator $- \Delta Ct$ sample

While $\Delta Ct$ calibrator $= Ct$ treated sample $- Ct$ refrence gene

and $\Delta Ct$ sample $= Ct$ treated sample $- Ct$ refrence gene.

The normalized treated sample amount in the sample was equal to $2^{-\Delta\Delta Ct}$ and this value could be used for comparing the expression levels in samples.

#### 2.3.3.4 RNA sec analysis

RNA extraction performed as described in section 2.3.1.2 but mixed with RNAstable® LD reagent to protect RNA from degradation and stored at room temperature. The RNAs contained three biological replicate for each stage of development and ripening (MG, 3 dpb and Ripe) in two genotypes of M82 and Q1968.

Gene expression data were generated at each of these fruit development stages to monitor changes in transcriptomics of pathways of interest. The extracted RNA was sent for Tomnet RHUL colleagues for RNA seq analysis. Analysis completed by: Junjian Ni, with great helps and supports from Josh Cohn, Suzy Stiegelmeyer, Julie Leonard and Pingsha Hu. The following steps were performed for this analysis.

Read Alignment, Data Normalization and Pre-processing:

RNA sequence data was generated for all eight samples and submitted to an internal pipeline for generating counts for RNAseq data. The pipeline incorporates the widely used alignment tool TopHat2. The software accounts for single end reads, which were used in this study, and merges the results into a single alignment file in the BAM format for each sample. Counts were generated from the BAM files using a script developed internally by Suzy Stiegelmeyer. The read counts per gene were generated as the number of reads aligning to each annotated gene region (transcription start site to transcription end site) extracted from tomato_v6_gene_models_plus_chloroplast.gff. This produced read count data for 34808 tomato genes. If a given read aligns to multiple genomic regions defined as genes, up to five regions received one count each. If the read aligned to more than five genomic loci defined as genes, then the read was discarded. Fastq sequences were examined by fastqc software, and all reads QC plots were accessed and reviewed through the following link (http://demeter.nafta.syngenta.org/project/RIC658/). Most of the samples had similar GC content consistent with the GC content of the tomato genome, with slightly bias to GC. The data were loaded into R and analysed using the EDASeq [2] package version 1.10.0. Within-lane normalization methods to correct for GC bias were used,

following by the normalization normalized using a full quantile between lane normalization.

Method Used for Differential Expression and Enrichment Analysis:

The normalized count was used for gene differential expression analysis via edgeR package. The mroast method in the limma and edgeR packages was used to determine if any Gene Ontology (GO) terms and pathways were significantly over represented in the data.

The analysed data transferred to RHUL. The data were classified and different types of altered genes were extracted from them. Top 20 differently expressed gene in the introgressed region and in the whole genome in different stages of ripening were extracted. the genes also classified based on their Go terms to different groups to have an over view about the differently expressed genes. The alteration of carotenoid biosynthesis pathway genes also were extracted and reported.

## 2.4 Plastid isolation and fractionation

Chloroplast and chromoplast fractionation were performed to determine potential membrane changes in CrtZW.DET1 line. Fresh tomato fruits in two developmental/ripening stages of ripening (mature green and 4 to 6 days post breaker) were harvested from selected plants, chopped and weighted (80 g). Samples were placed in a plastic bowl with an aluminum cover and stored overnight at 4°C to reduce the starch content. Cold extraction buffer (4°C, 1:3, w/v; 0.4 M sucrose, 1 mM DTT, 50 mM Tris, 1 mM EDTA, pH 7.8) was added to samples and left to infiltrate the tissue for 10 min. The mixture was then homogenized by three bursts from a Waring blender (Waring Products, UK). The mixture was filtered through 3-4 layers of muslin. The filtrate was transferred into two screw cap centrifuge tube (500 ml) and centrifuged in a pre-cold GSA-3 rotor at 5,000 *g* for 10 min at 4°C using a Sorval RC5C centrifuge (Thermo Scientific, UK). After centrifugation the supernatant was discarded and the pellet was resuspended in extraction buffer. Slurry was transferred into two 50 ml centrifuge tubes and balanced with extraction buffer. Centrifugation was performed at 4°C for 10 min at 9,000 *g* in a pre-cold GSA-5 rotor using a Sorval RC5C centrifuge (Thermo Scientific, UK). The supernatant was discarded and the pellet was dissolved in 45% sucrose buffer (45% w/v sucrose, 50 mM Tricine, 2 mM DTT, 5 mM sodium bisulphite, 2mM EDTA, pH 7.9; 3 ml). The chromoplasts/chloroplasts were broken by a hand held potter homogenizer (VWR,

UK). A typical volume of 8 ml in 45% sucrose buffer was obtained for the lysed plastid preparations. The slurry (4 ml) was transferred to two new 38.5 ml transparent Ultra-Clear™ centrifuge tubes (Beckmann Coulter, UK). Onto this suspension layers of buffer containing different sucrose concentrations were added including 38% w/v sucrose buffer (6 ml), 20% w/v sucrose buffer (6 ml), 15% w/v sucrose buffer (4 ml) and finally 5% w/v sucrose buffer (8 ml). After precise balancing with buffer, the tubes were centrifuged for 18 h at 100,000 *g* using a SW28 swing out rotor in a L7 ultracentrifuge (Beckman Coulter, UK). The tubes were removed from centrifuge and photographed to provide a visual record. Fractions (1ml) was collected from top layer of gradients using Minipuls®3 peristaltic pump and FC203B fraction collector (Gilson, UK). The fractions were transferred to labelled cap less microcentrifuge tubes (2 ml). Pigments were extracted as detailed in section 2.2.1.1 and stored at -20°C prior to UPLC-PDA analysis (section 2.2.3).

## 2.5 Hormone experiments

### 2.5.1 Abscisic acid (ABA) hormone measurement

#### 2.5.1.1 ABA extraction from fruit tissue

ABA was extracted from freeze dried tissue (detailed in section 2.1.3.3) following the protocol reported in (Fraser et al., 1995) with some modifications. Freeze dried tissue (50mg) was stirred for 15 h at 4°C in methanol/water (4:1, v/v; 2.5 ml). Samples were centrifuged at 4,000 rpm for 20 min at 4°C using a HERAEUS Pico17 centrifuge. The supernatant was pipetted to fresh 15 ml falcon tube and stored at 4°C. The pellet was re-extracted with methanol (1.5ml) for 3 h at 4°C. The filtrates were combined and divided into two screw-cap glass vials. Methanol was removed at 30°C using a GeneVac Ez-2 Plus rotary evaporator (UK). The aqueous residues were combined and the pH was adjusted to ~ pH 2.5 using pH indicator papers with 2 M HCl followed by partitioning against equal volume of ethyl acetate for 3 times. The combined ethyl acetate phase was taken to dryness using a GeneVac Ez-2 Plus rotary evaporator (UK). The residue was stored at −20°C prior to analysis using liquid chromatography-mass spectrometry (LC-MS).

### 2.5.1.2 LC-PDA-MS analyses of abscisic acid (ABA)

The extracted ABA sample (section 2.5.1.1) was eluted in water (MS grade, Fisher Scientific) and filtered using a 0.2 µm SYR FILTER NYL filter prior injection to LC-MS.

ABA Identification and quantification were carried out using the high resolution Q-TOF mass spectrometer UHR-MAXIS (Bruker Daltonics), connected to a UHPLC UltiMate 3000 equipped with a PDA detector (Dionex Softron). Chromatographic separations were performed on a YMC-UltraHTPro $C_{18}$ 2 µm column (100 x 2 mm i.d.) coupled to a 20 x 4.6 mm $C_{18}$ guard column was used (YMC Inc.). The mobile phase was comprised of (A) water, containing 0.05% formic acid and (B) acetonitrile, containing 0.05% formic acid. These solvents were used in a gradient mode starting at 95% (A), stepped to 50% (A) for 18 min and followed by a linear gradient over 2 min to 20% (A). This last condition was kept for 4 min in isocratic mode and after that initial conditions (95% A) were restored for 1 min. The column was re-equilibrated for 10 min. The used flow rate was 0.2 ml/min and the injection volume was 5 µl. Electrospray ionisation (ESI) was carried out in negative mode. Dry gas (nitrogen) and nebulizer were set at 8 l/min and 1.3 bar respectively, and drying gas temperature was set at 195 °C. Capillary voltage was used at 3.5 kV and a full MS scan was performed from 50 to 1200 *m/z*. Instrument calibration was performed externally prior to each sequence with sodium formate solution (10 mM). Automated post-run internal calibration was performed by injecting the same sodium formate calibrant solution at the end of each sample run via a six port divert valve equipped with a 20 µl loop.

Raw data files were processed with the manufactures software Compass Data Analysis version 4.0 (Bruker Daltonics). Areas of ABA peaks eluting at 15.3 min were integrated from extracted ion chromatograms at 263.1278 and used for quantification purposes.

Different known concentrations (2ng to 10µg) of ABA standard were used for creating the ABA dose response curve.

### 2.5.2 Ethylene hormone measurement

Fruits at defined stages of ripening were harvested, weighted and placed in gas-tight jars (250 ml) containing a Precision Seal® rubber septa in the lid. The lid was sealed with parafilm and stored for 12 h at room temperature. A sample of headspace (5 ml)

gas was injected to a ProGC gas chromatograph equipped with a Flame Ionization Detector (FID) and separation column SPEC-06774. Nitrogen and air were used as the carrier gas with 2 bar flow rate. $H_2$ were used as fuel. An ethylene standard curve was created by injecting different quantities of the ethylene authentic standard (different concentrations of ethylene were prepared from 1.01% ethylene standard). Subsequently the sample's areas were compared to standard curve and normalized for fruit weigh and storage parameters.

## 2.6 Microscopy

### 2.6.1 Slide preparation

#### 2.6.1.1 Cell slide preparation

Mature green and ripe fresh fruit were harvested and chopped to small pieces. Samples were fixed immediately in glutaraldehyde (3.5%, v/v) for 1 h in darkness followed by a 20 min incubation in EDTA solution (0.1 M, pH 9) at 60°C for mature green samples and at room temperature for ripe samples (Enfissi et al., 2010). Afterwards the solvent was decanted and pericarp samples (which were softened after treatments) were placed and spread on a microscope slide using a spatula. A drop of distilled water was added to the samples. The cover slide (22 x 22 mm) was placed and the samples were kept wet prior to viewing. Samples were observed within some hours using a Nikon H600L microscope (detailed in section 2.6.2).

#### 2.6.1.2 Cuticle slide preparation

Mature green fruits were collected and very thin layers of pericarp (~50 sample for each biological rep) were prepared using a sharp razor blade. Sudan IV/propanol stock solution (0.1%, w/v) was diluted (3:2 v/v) with distilled water. The solution was mixed using a vortex and centrifuged at 10,000 rpm for 10 min to remove precipitant using an Eppendorf centrifuge 5810R. The upper layer of solution was transferred to 2 ml microcentrifuge tubes containing pericarp samples and stored at room temperature for 10 min, followed by two washing steps. The samples were rinsed firstly with propanol/distilled water (50%, v/v) and subsequently with distilled water (Buda et al., 2009). Samples were placed carefully on microscope slides using two forceps. Some drops of distilled water were added to samples and the cover slides (22

x 22 mm) were placed. Samples were kept wet prior to viewing. All samples were viewed within 1 to 2 hours after preparation.

### 2.6.2 Nomarski microscopy

Prepared slides (sections 2.6.1.1 and 2.6.1.2) were imaged using Nomarski differential interference contrast optics and florescence optics on a Nikon H600L microscope coupled with a Nikon DS-Fi2 colour camera. NIS Elements software (version 4.2) was used for microscope operation. All cells and chloroplast areas, chloroplast numbers per cell and cuticle thickness were measured using NIS Elements version 4.2 software. All measurements done for at least three biological replicates as explained in section 2.1.1 with 12 measurements through different parts of fruit for each biological replicate.

## 2.7 Cell component measurement

### 2.7.1 Extraction of alcohol-insoluble solids (AISs)

Crude cell wall sample (alcohol-insoluble solids (AISs)) was extracted for subsequent cell wall composition measurements. All extraction processes were performed using the protocol described in (Huysamer et al., 1997) with some modifications.

Frozen tissues (detailed in section 2.1.3.1; 5 gr) were boiled in absolute ethanol for 30 min for inactivating autolytic enzymes. Samples were transferred to a 50 ml falcon tube and homogenized using a Brinkmann Polytron Homogenizer followed by a centrifugation at 4,000 rpm for 10 min using a HERAEUS Pico17 centrifuge. The supernatant was discarded and the pellet washed twice with ethanol and then two acetone washes. At each washing stage in the protocol, samples were centrifuged at 4,000 rpm for 10 min. The pellet (crude cell wall) was dried overnight in a fume hood and then stored in a desiccator. The weight of pellet was reported as dry weight of each sample.

### 2.7.2 Uronic acid measurement

Water-soluble uronic acids were extracted from AISs using the protocol described by (Campbell et al., 1990). AIS (5 mg) was stirred in distilled water (0.5 ml) for 3 h using a rotary invertor. Samples were then centrifuged at 14,000 rpm for 10 min using an Eppendorf centrifuge 5810R. The supernatant was transferred to new 1.5 ml microcentrifuge tube and dipped in liquid nitrogen prior to freeze drying for 10 h. the weight of residue was recorded.

In the next step a specific colorimetric assay was performed for the detection of uronic acid content in the samples (Blumenkrantz and Asboe-Hansen, 1973). The freeze-dried residue from previous stages were dissolved in distilled water (300µl) and transferred to 10 ml Pyrex screw-cap test tubes. Fresh Sulfuric acid/tetraborate solution (75mM; 1.2 ml) was added to sample and refrigerated on crushed ice. Tubes were mixed and heated in a water bath at 100°C for 5 min. After cooling on crushed ice, 3-phenylphenol (9 mM; 20µl) was added and mixed using a vortex. The absorbance of sample was detected at 520 nm using a DU®800 Spectrophotometer (Beckman Coulter, UK). A blank sample was run with samples and used for zeroing the spectrophotometer. Standard curve of uronic acid was created by measuring the absorbance of different known concentrations of galacturonic acid standard.

### 2.7.3 Cellulose measurement

Cellulose measurement was performed based on cellulose resistance to dilute acids and its insolubility in water (Kulic and Radojicic, 2011). AIS (10 mg) was weighted to 10 ml Pyrex screw-cap test tubes. A mixture of nitric acid/acetic acid (1:10, v/v; 2ml) was added to sample and boiled for 10 min at 120°C under fume hood. The sample was cooled down, centrifuged at 2,000 rpm for 10 min using a HERAEUS Pico17 centrifuge and the supernatant discarded. Two washing steps with ethanol and acetone respectively were performed. The weight of dried cellulose residue was obtained by deducting the weight of sample tube after and before experiment.

## 2.8 Enzyme assays

### 2.8.1 Enzyme extraction

Enzyme extraction was performed as detailed previously for subsequent analysis (Eriksson et al., 2004). Frozen pericarp tissues (section 2.1.3.1; 10 gr) were homogenized with 30 ml of cold distilled water using a Brinkmann Polytron Homogenizer. All subsequent steps were performed at 4°C in the cold room. Homogenized samples were stirred for 30 min on a rotary invertor. NaCl was added to slurry to a final concentration of 1.0 M. The pH was adjusted to pH 6 using 0.5 M NaOH, followed by 1 h of stirring. The sample was centrifuged at 4,000 rpm for 30 min at 4°C using a HERAEUS Pico17 centrifuge. Supernatant transferred to new falcon tube (50 ml) and ammonium sulfate was added to a final concentration of 80% of saturation. The precipitated proteins were collected by centrifugation. The pellet

was re-suspended in NaCl solution (0.15 M, pH 6; 3 ml) and dialyzed against 0.15 M NaCl overnight. The final volume of all extractions reached 6 ml and then aliquoted prior to storage at −20°C and used for subsequent enzyme assays.

### 2.8.2 Polygalacturonase assay

Polygalacturonase activity was measured using a colorimetric assay specific for the enzyme (Tucker et al., 1980). Crude extracts from frozen samples were prepared (section 2.8.1). Polygalacturonic acid solution (0.5%, w/v; in 50 mM sodium acetate buffer, pH 5 at 30°C; 1.9 ml) was transferred to 10 ml Pyrex screw-cap test tubes and the diluted enzyme extract (1:3, v/v, in 0.15 M NaCl pH 6; 100µl) was added. The buffer used to prepare the extracts (0.15 M NaCl pH 6; 100µl) was added instead of crude enzyme extract for blank samples. Sample was mixed immediately by inversion and incubated at 30°C for 10 min using a water bath. Copper solution (16 mM copper sulfate, 1300 mM sodium sulfate, 226 mM sodium carbonate, 190 mM sodium bicarbonate and 43 mM potassium tartrate solution; 2 ml) was added to tube, followed by another incubation at 100°C for 15 min using a water bath. The tube containing the enzyme assay mixture was cooled down to room temperature by refrigerating on crushed ice. Arsenomolybdate solution (40 mM molybdic acid, 19 mM arsenic acid and 756 mM sulfuric acid; 2 ml) was added to sample and mixed by inversion until foaming stopped. The tube was then centrifuged for 15 min at 2000 rpm using a HERAEUS Pico17 centrifuge. The supernatant was transferred to cuvette and the sample absorbance was recorded at 450 nm for both blank and sample using a DU®800 Spectrophotometer (Beckman Coulter, UK). Standard curve of D-galacturonic acid was plotted using different known concentrations of standard. One unit of enzyme was defined as 1.0 µmol of released reduced sugar, measured as D-galacturonic acid from poly-galacturonic acid per min at pH 5.0 at 30°C.

### 2.8.3 β-galactosidase assay

β-galactosidase activity was detected by a colorimetric assay, known to be specific for the enzyme (Carey et al., 1995). Crude enzyme preparations were generated from frozen samples (section 2.8.1). *Ortho*-Nitrophenyl β-D-galactopyranoside is a colourless substrate, which when acted on by the β-galactosidase releases galactose and *Ortho*-Nitrophenyl which has a yellow color and is thus used to indicate enzyme activity. The substrate *Ortho*-Nitrophenyl β-D-galactopyranoside (13 mM; 400µl) was transferred to 10 ml Pyrex screw-cap test tubes. Subsequently citrate solution (0.1 M,

pH 4; 500µl), BSA (0.1%; 0.4) and diluted enzyme (1:3, v/v, diluted in 0.15 M NaCl pH 6; 100µl) were added to tubes and mixed by swirling prior to incubation at 37°C for 10 min. The reaction was terminated by addition of sodium carbonate (0.2 M; 2ml) to samples. The liberated *Ortho*-Nitrophenyl was measured at 415 nm using a DU®800 Spectrophotometer (Beckman Coulter, UK). The concentration of galactose was determined using extinction coefficient of *Ortho*-Nitrophenyl at 415nm. One unit of enzyme was defined as the amount of enzyme that hydrolyzes 1 µmol of *Ortho*-Nitrophenyl β-D-galactopyranoside in 1 min.

### 2.8.4 Pectinesterase assay

Pectin esterase activity was measured using a titrimetric assay with some modification (Kertesz, 1995, 1957). Crude cell-free extracts were prepared from frozen samples (section 2.8.1). Pectin solution (1%, w/v, in 100 mM NaCl; 20 ml) was added to a titration vessel in conjunction with a heater-stirrer using a Heat/Stir STUART. A pH meter was used for recording the pH changes during experiment. All steps were performed at 30°C. Pectin solution was adjusted to pH 7.5 with sodium hydroxide solution (20 mM). The volume of sodium hydroxide solution and the time required for obtaining pH 7.5 were recorded. The Diluted enzyme solution (1:3, v/v, in 1.7 M NaCl; 200µl) was added to vessel and the reaction was run for 3 min. Subsequently the tubes were incubated for 15 min at 100°C for the enzyme inactivation. The reaction was stopped using a water bath. The pH of reaction was maintained at 7.5 by adding sodium hydroxide solution. The used volume of solvent and the required time were recorded. Unit of enzyme was calculated using the below equation:

$$Unit\ per\ ml\ Enzyme = \frac{\frac{ml\ NaOH\ for\ Test}{T1} - \frac{ml\ NaOH\ for\ blank}{T2}(1000)(df)}{(Tt)(\text{Vol enz})}$$

T1 = Required time to maintain pH 7.5 for test

T2 = Required time to maintain pH 7.5 for blank

Tt = Total run time of reaction

df = Dilution factor of enzyme

Vol enz = Volume of used enzyme in each reaction

Calculated unit of enzyme was normalized for gram of frozen tissue. One unit of enzyme will release 1 micro equivalent of acid from pectin per min at pH 7.5 at 30°C.

## 2.9 Shelf life experiment

Shelf-life experiments were performed using ripe fruits. At least 9 ripe fruits were harvested for each experiment. The weight and firmness (see section 2.1.2) of fruits were recorded prior to sterilization in 0.5% bleach for 10 min for providing a sterile and equal condition for all of the samples. Fruits were rinsed three times with sterile distilled water and air-dried under sterile fume hood. Samples were transferred to sterile Phytatray™ containers and stored at 19°C in dark. The weight and firmness factor of samples were recorded regularly. The old container was replaced with a new sterile container after each measurement. Three fruits were collected as detailed in section 2.1.3.3 for further analysis at 30, 50, 85 days post harvest and the end stage was defined at the time that fruit firmness reached to ~ 0% of Fff.

## 2.10 Statistical analysis

A minimum of three biological and three technical replicates were used for each experiment unless stated otherwise (see section 2.1.1). For enzyme and cell wall component experiments, starting tissue was a mixture of three biological samples and three biological samples at each stage unless stated otherwise.

Based on experiment type, Student's t-test (Dunnett's test) or Anova were performed for indicating the significant differences between samples. P value indicates the degree of differences. Lower P value indicates more difference which $P < 0.05$, $P < 0.01$, and $P < 0.001$ are indicated by *, **, and ***, respectively.

PCA was performed on metabolite data matrices. SIMCA-P+ software v. 13.0.2 (Umetrics, UK) was used to display PCA analysis.

# CHAPTER 3: IDENTIFICATION, VALIDATION AND CHARACTERISATIONS OF TRANSCRIPTIONAL REGULATORS OPTIMISING PIGMENTATION IN PLANTS

## 3.1 Introduction

There are several important factors that are involved in the control of fruit ripening. These include plant hormones such as ethylene as well as environmental factors which include temperature and light (Dumas et al., 2003). The effect of transcription factors during fruit development and ripening is a very important aspect of regulation that has the potential to influence important key steps in ripening.

Considering the importance of TFs in regulating the fruit development and ripening processes, efforts have been focused on deciphering the associated transcriptional cascades using modern technologies associated with functional genomics and multi parallel omics studies. In this section of the study the work performed has been focused on the validation and characterisation of putative transcription factors affecting fruit ripening and pigmentation in fruits.

*APRR2* transcription factor [ARABIDOPSIS PSEUDO-RESPONSE REGULATOR-2] is an important TF affecting fruit development and ripening (Fitter et al., 2002). In tomato *APRR2* is shown that affect the accumulation of pigments in fruit. *APRR2-Like* TF in tomato genome assembly (The Tomato Genome Consortium) was designated as Solyc08g077230 (Pan et al., 2013). *APRR2-Like* is located on chromosome 8. In the first objective of first aim in this chapter search for any other possible homologue of this gene on other chromosomes and its potential effects on pigmentation and ripening mechanism is targeted (see section 3.2.1).

In the second objective of first aim, some candidate TFs derived from Artificial Neural Networks and Correlation Expression which named Exploiting Systems Biology (ESB); ESB-LINK project outputs have been transformed to stable knock-down transgenic lines (contact person Prof Seymour, University of Nottingham). These TFs are candidate genes for some tomato ripening regulators. These stable transgenic lines are downstream TFs derived from the master regulator RIN, which are candidate genes potentially affecting fruit colour, flavour and texture. ESBs transgenic lines created in result of RHUL and Syngenta Company collaborations. The construct was generated internally by Syngenta Ltd and it represents a constitutive RNAi approach. The ESB9 is designated as Solyc06g053960 ID in tomato genome assembly (Sol network) and with the Le number of Le007684. In this chapter characterising the function of this gene is the aim (see sections 3.2.2.1, 3.2.2.2, 3.2.2.2.1 and 3.2.2.2.2).

Systems Biology and QTL mapping has identified a number of regulatory genes associated with fruit pigmentation (Fraser et all., unpublished). The SubIL of 2-3/2-4 from *S. pennellii,* designated Q1968 was found to have high lycopene content compared to the wild type counterpart. This region underlying two putative TFs. Therefore, in the third objective of first aim the question is: are these two TFs involved in the accumulation of lycopene in Q1968 line? Different strategies like metabolomics and transcriptomics have been performed to investigate the potential effects of these two TFs on carotenoid production (see sections 3.2.3.1, 3.2.3.2, 3.2.3.3 and 3.2.3.4).

## 3.2 Results

### 3.2.1 Validation of *APRR2-Like* homologue gene

*APRR2-Like* TF in tomato genome assembly (The Tomato Genome Consortium 2012) was designated as Solyc08g077230 (Pan et al., 2013). *APRR2-Like* gene is located on chromosome 8 and its candidate homologue in chromosome 6 was found in database searches which could be a potential effective TF on fruit pigmentation. The initial search in this study for finding the homologue has performed in 2012 and this gene aligned with *APRR2-Like* gene strongly but as far as its mRNA was truncated (430 nucleotides in comparison to *APRR2-Like* mRNA that was 2233 nucleotides), it seemed to be a pseudo gene. Therefore, this part of project was discarded for further work. However, in subsequent versions of the tomato genome assembly (The Tomato Genome Consortium 2015), the updated data indicate 2053 nucleotide for mRNA of the homologue. This gene seems a proper homologue for *APRR2-Like* gene as the length of its mRNA was close to the *APRR2-Like* mRNA with 2233 nucleotides. Further characterisation of this gene is needed for future works. The cDNA of *APRR2-Like* gene and its homologue in chromosome 6 and also their alignment are shown in Appendix 3 Figure A 9-1 and Appendix 4 Figure A 9-2.

### 3.2.2 ESB9 lines primary characterisation

#### 3.2.2.1 ESB9 lines phenotype characterisation

In this chapter we aim to investigate the potential effect of ESB9 TF on fruit quality factors for instance fruit pigmentation and ripening process. ESB9 line is an RNAi knock down stable transgenic line for a heat shock transcription factor-like with a

direct *RIN* binding site on its promoter (Seymour et all., unpublished). Phenotypic variation of primary ESB9 plants from AC comparator and also leaf and fruit pigment analysis were carried out as a part of this chapter. Eleven RNAi knock down transgenic lines of ESB9, were used for phenotype characterisation. During the plants growth cycle, the ESB9 physiological phenotypes were monitored by different experiments. The visual change observed was leaf colouration compared to the AC control line. Paler leaf colour observed in 9 out of 11 lines of ESB9. With the exception of lines 6 and 11, the lines displayed a light green colour in comparison to the dark green colour of the leaf material from the AC control line (see Figure 3-1). For this phenotypic differences UPLC pigment analysis was subsequently performed (see section 3.2.2.2).

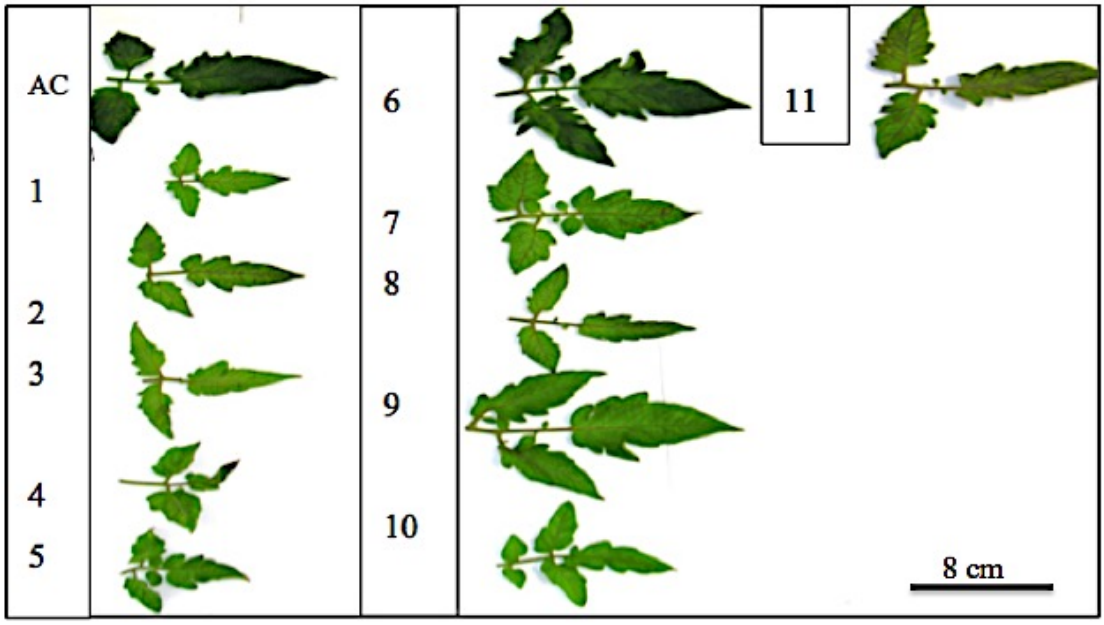



Figure 3-1 Phenotypic leaf colour differences in 11 primary transgenic ESB9 (1-11) lines and Ailsa Craig (AC) as control line.

Expanding leaves for each line were harvested. Younger leaves, at the top of the plants, were preferentially chosen. The scale bar represents 8cm. As shown in the picture the leaf size of different lines were different because of physiological differences among lines.

Existence of ESB9 transgene was confirmed by normal PCR of NptII gene (Neomycin phosphotransferase II gene). NptII gene is responsible for the gene products that confer Kanamycin resistance. Gel electrophoresis confirms the existence of NptII amplicon (see Figure 3-2). All of 11 primary transgenic lines were positive for the NptII gene, which suggests the plants have been successfully transformed.

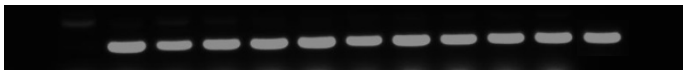



Figure 3-2 PCR confirmation of the presence of transgene in the 11 ESB9 lines and its absence in AC control line.

Amplification of *NptII* gene was performed by PCR on the DNA pool sample of 3 biological replicates of the each ESB9 line and the control line. Gel results visualised under UV light. NTC: non-template control; AC: Ailsa Craig Control line.

Firmness changes are another important phenotypic parameter for characterizing fruit softening and related ripening processes. Firmness measurements (see section 2.1.2) were performed for ESB9 lines and AC at the MG and ripe stages. The significant changes are indicates with stars in the tables (see Table 3-1) as explained in section 2.10; and only the significant changes are reported as increases and decreases in this study. At the MG stage firmness was almost the same in 7 of the ESB9 lines compared to the AC control. Firmness increased slightly in 3 plants and in one of them decreased by 10% (see Table 3-1).

As Table 3-1 illustrates in 3 ESB9 plants the firmness increased more than 15% and in other three plants the firmness is around 14% less than AC average firmness at ripe stage. In other 5 of plants firmness is nearly the same as the control AC.

To sum up firmness changes do not show a consistence change and in most of plants they are similar to the control lines.

Table 3-1 Firmness results of control line (AC) and 11 ESB9 transgenic lines (1-11) in mature green stage of ripening

The table indicate the percentage of Fruit firmness factor (Fff) in control line (AC) and in 11 ESB9 lines. Measurements were made from three biological replicate with three technical replicate in AC and from each independent biological sample with three technical replicates in ESB9 lines. The ±SD is shown as Error bars. Dunnett's test was used for determination of significant differences between the wild type control lines (AC) and the 11 ESB9 lines. Significant differences of 11 lines to AC control line are shown by stars above columns which *, ** and *** are P<0.05, P<0.01 and P<0.001 respectively.

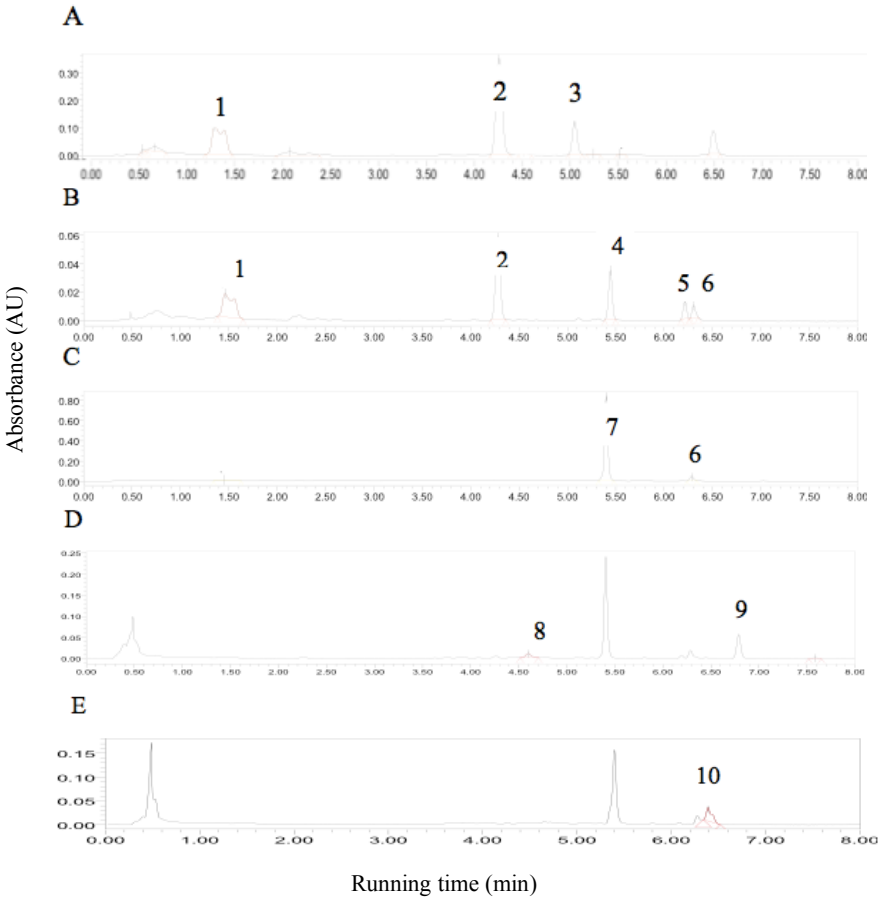
| | **AC** | **Line 1** | **Line 2** | **Line 3** | **Line 4** | **Line 5** | **Line 6** | **Line 7** | **Line 8** | **Line 9** | **Line 10** | **Line 11** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Mature Green** | 98 ± 0.94 | **87.67 ± 1.7** ** | 98.67 ± 1.25 | 97.67 ± 0.47 | 92.67 ± 3.09 | **99.67 ± 0.47** * | 97.33 ± 1.7 | 99 ± 0.82 | 98.33 ± 0.47 | **99.33 ± 0.47** * | 98.67 ± 0.94 | **99.67 ± 0.47** * |
| **Ripe** | 61.89 ± 5.51 | **53.33 ± 1.25** ** | 63.33 ± 2.36 | 63.33 ± 3.4 | **54.33 ± 1.25** ** | 58.67 ± 4.78 | 67 ± 2.45 | **52.33 ± 1.89** ** | **72.33 ± 2.05** ** | **69.67 ± 2.87** * | 63.67 ± 2.49 | **72 ± 3.27** * |

#### 3.2.2.2 Assessment of carotenoid profiles in leaves and tomato fruits during fruit development and ripening

##### 3.2.2.2.1 Assessment of pigment characteristics based on UPLC spectrum

In order to assess the pigment changes in all of the projects, chromatographic analysis of carotenoids, chlorophylls and α-tocopherol were carried out as a part of most of projects (ESB9, Q1968, ZW.DET1, ZW.APRR2 and shelf life experiments). Extracted leaf and fruit samples were analysed under Ultra and High Performance Liquid Chromatography (UPLC-PDA) to have a better understanding about the level of pigments in each line and its control for detecting pigment profile changes. The UPLC method is described in section 2.2.3. Figure 3-3 illustrates sample chromatogram profiles of pigments and their spectral characteristics of M82 line. Based on the program, which is used for UPLC, the retention time of eluted pigments varies. Different wavelengths were investigated for variety of carotenoids, α-tocopherol, chlorophylls and its degraded forms of pheophytin.

Lutein, chlorophylls, pheophytin and β-carotene were detectable in mature green fruits and leaves at 450 nm. In ripe fruits lutein, lycopene and β-carotene are observed at λ max of 450 nm, while α-tocopherol and phytoene are available in 286 nm and phytofluene exist is 350 nm. See Figure 3-3 for more details about the retention time, exact spectra for each pigment and related peaks on chromatographs.




F

| Names | Peak number | Retention time (min) | Spectra $\lambda_{max}$ (nm) |
|---|---|---|---|
| Lutein | 1 | 1.45 | 448.0, 476.0 |
| Chlorophyll b | 2 | 4.28 | 456.5 |
| Chlorophyll a | 3 | 4.95 | 429.8 |
| Pheophytin b | 4 | 5.44 | 434.7 |
| Pheophytin a | 5 | 6.2 | 409.3 |
| β-Carotene | 6 | 6.29 | 454.1, 479.6 |
| Lycopene | 7 | 5.39 | 472.3, 502.7 |
| α-Tocopherol | 8 | 4.6 | 293.4 |
| Phytoene | 9 | 6.77 | 286.2 |
| Phytofluene | 10 | 6.44 | 349.0, 368.3 |

Figure 3-3 Chromatographic profiles and spectral characteristics of carotenoids, chlorophylls and α-tocopherol in M82 line analysed by UPLC.

A. Leaf chromatograph of M82 at 450 nm, B. Fruit chromatograph of M82 in mature green stage at 450 nm, C. Fruit chromatograph of M82 in ripe stage at 450 nm, D. Fruit chromatograph of M82 in ripe stage at 286 nm, E. Fruit chromatograph of M82 in ripe stage at 350 nm, F. The table illustrates the spectral characteristics of pigments (recorded from 250 to 600 nm) using UPLC, retention time and the spectra for each pigment is shown and peak numbers are indicated on chromatographs.

#### 3.2.2.2.2 Best line selection through first generation pigment profile for ESB9 plants

In addition to phenotypic characterisation of ESB9 plants, pigment profiles of these 11 transgenic plants also were analysed. UPLC-PDA analysis was performed as described in section 2.2.3. Leaf, mature green fruits (MG) and 7 dpb fruit samples were analysed for an initial insight into the effect of knocking down the gene designated *ESB9* on carotenoids and chlorophylls profile. Pigment profile of leaf, MG fruits and 7 dpb fruits are provided in Table 3-2, Table 3-3 and Table 3-4 respectively. Some changes were detected in the chlorophyll a, chlorophyll b, lutein and β-carotene content in the leaf material from a proportion of the lines. As it is shown on Table 3-2 chlorophyll a content in leaf material from the ESB9 lines decreased around 30%-70% in comparison to the AC control in 5 lines and is higher in 2 of the lines, while the rest showed no change. Chlorophyll b decreased around 15%-60% in 7 lines out of 11, which is a significant change in leaves from most of the lines. Overall, chlorophylls showed the highest alterations in comparison to other pigments in leaves. In MG fruits some increases and decreases were observed in chlorophylls and carotenoids levels. Pheophytin a & b, which represents degraded forms of chlorophyll a & b, decreased significantly in 6 and 7 lines out of 11 ESB9 lines when compared to the AC control respectively. Four of ESB9 lines showed lower chlorophyll b content but in 2 lines out of 11, chlorophyll a increased. β-Carotene content in 5 of ESB9 lines was lower between 6%-35%, but higher in 2 lines and in 4 of the lines did not show any changes (see Table 3-4).

As expected for the *ESB9* gene, which from gene regulatory networks is a downstream TF linked to the master RIN regulator which has a vast effect on ripening (Fujisawa et al., 2013), most of these considerable changes occurring at 7 dpb (ripe) stage. Mostly the carotenoid levels decreased at this stage. As presented in Table 3-4 a reduction in phytoene, lycopene, lutein and β-Carotene contents were observed in a proportion of the ESB9 lines. Just 5 plants from 11 plants of ESB9 present a significant change in lycopene level and these 5 lines showed 50% to 75% decrease in lycopene content. Interestingly, all 11 ESB9 lines had reduced β-Carotene content (55%- 83%) at the ripe stage of fruit development. This considerable change in all of the ESB9 lines could be a valuable result for the better understanding of the *ESB9* gene. In general from 11 primary plants containing *ESB9* gene, total carotenoid levels decreased in 5 lines and 6 of plants did not show any changes. For introducing the

best lines containing the most pigment level changes, ESB9-2, ESB9-3 and ESB9-8 ,which had a decrease in all of carotenoid contents at ripe stage, were chosen for more investigation. Better characterisation of this gene can help for improvement of carotenoid levels which depends on next generation analyses. For example, making an over expressed transgenic plants of this gene can improve the carotenoid levels. But as the limited time of this study did not let us go further, the functional characterisation of the ESB9 gene in the first generation of transgenes were provided to our collaborating partners to continue with.

Table 3-2 Carotenoids and Chlorophylls contents in leaves of wild type Ailsa Craig (AC) and 11 ESB9 lines.

Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each ESB9 samples were made from pool of three leaves for each line. Each sample had three technical replicates. AC samples were made from independent pools of minimum three leaves from three plants. The mean data are shown with ± SD. Dunnett's test was used to determine significant differences between the background (AC) and ESB9 lines. Significant differences of 11 lines compared to AC control line are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

| | **AC** | **Line 1** | **Line 2** | **Line 3** | **Line 4** | **Line 5** | **Line 6** | **Line 7** | **Line 8** | **Line 9** | **Line 10** | **Line 11** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chlorophyll a** | 334 ± 10 | 323 ± 18 | **239 ± 7*** ** | 256 ± 30 | **260 ± 16*** | 290 ± 23 | **173 ± 9*** ** | **237 ± 11*** ** | 361 ± 20 | **455 ± 10*** ** | **411 ± 9*** ** | **111 ± 14*** ** |
| **Chlorophyll b** | 867 ± 88 | **708 ± 42*** | **712 ± 30*** | **739 ± 47*** | **743 ± 23*** | 786 ± 32 | **576 ± 2*** ** | **478 ± 24*** ** | 839 ± 30 | **974 ± 19*** | **1023 ± 45*** | **369 ± 10*** ** |
| **Lutein** | 159 ± 19 | 158 ± 11 | 151 ± 6 | 165 ± 12 | 170 ± 4 | 173 ± 5 | **131 ± 3*** | **109 ± 6**** | 170 ± 9 | **204 ± 8**** | **224 ± 13**** | **72 ± 2*** ** |
| **β-Carotene** | 88 ± 14 | 93 ± 7 | 87 ± 3 | 94 ± 8 | 94 ± 4 | 98 ± 4 | **77 ± 1** | **56 ± 3**** | 87 ± 4 | **106 ± 5*** | **122 ± 9**** | **37 ± 2*** ** |
| **Total Carotenoids** | 247 ± 31 | 251 ± 18 | 238 ± 10 | 259 ± 20 | 265 ± 7 | 271 ± 9 | **208 ± 3*** | **165 ± 10**** | 257 ± 13 | **310 ± 13**** | **346 ± 21**** | **109 ± 4*** ** |

Table 3-3 Carotenoids and Chlorophylls contents in mature green fruits of wild type Ailsa Craig (AC) and 11 ESB9 lines.

Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each ESB9 samples were made from pool of three fruits for each line. Each sample had three technical replicates. AC samples were made from independent pools of minimum three fruits for each of three plants. The mean data are shown with ± SD. Dunnett's test was used to determine significant differences between the background (AC) and ESB9 lines. Significant differences of 11 lines compared to AC control are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

| | AC | Line 1 | Line 2 | Line 3 | Line 4 | Line 5 | Line 6 | Line 7 | Line 8 | Line 9 | Line 10 | Line 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chlorophyll a** | 12 ± 3 | 12 ± 1 | 9 ± 1 | 11 ± 0 | 11 ± 1 | 8 ± 1 | 15 ± 4 | 16 ± 3 | **45 ± 1**** | **32 ± 4**** | 20 ± 5 | 0 |
| **Chlorophyll b** | 152 ± 17 | **242 ± 1***** | 150 ± 38 | **107 ± 14*** | **180 ± 11*** | **106 ± 8**** | 127 ± 10 | 146 ± 7 | **215 ± 9***** | 158 ± 3 | **194 ± 9**** | 0 |
| **Pheophytin a** | 8 ± 1 | **10 ± 0**** | 7 ± 1 | **5 ± 0***** | 8 ± 0 | **5 ± 0**** | **6 ± 0***** | **6 ± 0**** | **6 ± 0**** | **5 ± 0***** | 8 ± 0 | **6 ± 0**** |
| **Pheophytin b** | 10 ± 4 | 7 ± 0 | 6 ± 1 | **4 ± 0*** | 5 ± 0 | **4 ± 1*** | **4 ± 0*** | **4 ± 0*** | **4 ± 0*** | **3 ± 0*** | 5 ± 1 | **26 ± 1***** |
| **Lutein** | 28 ± 3 | **44 ± 1***** | 32 ± 6 | **21 ± 2*** | **34 ± 2*** | 25 ± 2 | 31 ± 2 | 31 ± 1 | **46 ± 2***** | 32 ± 1 | **39 ± 1***** | 27 ± 1 |
| **β-Carotene** | 18 ± 1 | **23 ± 0***** | 18 ± 3 | **12 ± 1***** | 18 ± 0 | **14 ± 1**** | **15 ± 1**** | **17 ± 1*** | **22 ± 1*** | 18 ± 0 | **21 ± 1*** | **12 ± 1***** |
| **Total Carotenoids** | 47 ± 4 | **67 ± 1***** | 50 ± 9 | **34 ± 2**** | **52 ± 2*** | **39 ± 3*** | 46 ± 2 | 48 ± 2 | **69 ± 3***** | 49 ± 1 | **60 ± 2**** | **39 ± 2*** |

Table 3-4 Carotenoids and Chlorophylls contents in 7 days post breaker fruits of wild type Ailsa Craig (AC) and 11 ESB9 lines

Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each ESB9 samples were made from pool of three fruits for each line. Each sample had three technical replicates. AC samples were made from independent pools of minimum three fruits from three plants. The mean data are shown with ± SD. Dunnett's test was used to determine significant differences between the background (AC) and ESB9 lines. Significant differences of 11 lines compared to AC control are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

| | AC | Line 1 | Line 2 | Line 3 | Line 4 | Line 5 | Line 6 | Line 7 | Line 8 | Line 9 | Line 10 | Line 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phytoene** | 155 ± 31 | **105 ± 5*** | **81 ± 5**** | **44 ± 2***** | 167 ± 7 | 167 ± 10 | **87 ± 22*** | **261 ± 12***** | **56 ± 2***** | 152 ± 3 | 142 ± 6 | **201 ± 4*** |
| **Lycopene** | 1111 ± 378 | 907 ± 21 | **555 ± 40*** | **277 ± 97**** | 933 ± 40 | 1071 ± 21 | **583 ± 37*** | 1444 ± 85 | **429 ± 28**** | **454 ± 57*** | 926 ± 33 | 1149 ± 67 |
| **Lutein** | 35 ± 10 | 31 ± 3 | **19 ± 1*** | **21 ± 1*** | **23 ± 3*** | 23 ± 5 | 31 ± 7 | **18 ± 3*** | **51 ± 1*** | 33 ± 4 | 31 ± 1 | **17 ± 6*** |
| **β-Carotene** | 237 ± 16 | **77 ± 7***** | **42 ± 3***** | **49 ± 1***** | **46 ± 3***** | **72 ± 7***** | **85 ± 22**** | **60 ± 3***** | **101 ± 6***** | **109 ± 9***** | **65 ± 10***** | **80 ± 6***** |
| **Total Carotenoids** | 1538 ± 404 | 1121 ± 27 | **513 ± 268**** | **391 ± 95***** | 1170 ± 48 | 1333 ± 39 | **787 ± 87**** | 1783 ± 94 | **637 ± 35**** | **749 ± 61**** | 1154 ± 49 | 1447 ± 71 |

### 3.2.3 Characterisation of Q1968, the putative high lycopene line

#### 3.2.3.1 Q1968 phenotype characterisation

From crossing between two SubILs of 2-3 and 2-4 of *S. pennellii* of M82 background, a new derived SubIL was identified and designated as Q1968 (SubIL of 2-3/2-4 from *S. pennellii*). These results came out from previous projects in the lab in collaboration with Syngenta Ltd. and Nottingham university. The tomato genome sequence has revealed two putative transcription factors out of 64 genes were located within the introgressed genomic region. These two TFs were designated Solyc02g081780.1 and Solyc02g082040.2 under Sol Genomic Network (http://solgenomics.net). These genes encode a B3 family protein containing REM9-Like protein, and a *Myb-Like* transcription factor respectively.

Tomato seeds from lines containing the Q1968 and its isogenic control M82 were used to characterise the sub-line designated Q1968. Phenotypic differences were detected between two lines. At a similar developmental stage, from visual observations the Q1968 line was observed as produce bigger plants in size (see Figure 3-4), more fruits but a reduced number of seeds. These observations were not quantified and need more detailed data for support.

A


B


Figure 3-4 Phenotypic differences observed with the Q1968 variety compared to the M82 comparator

A. M82; B. Q1968. The representative pictures were taken in the same stage of plant growth. The seeds of all plants were sowed in the same batch and grown in the same greenhouse condition. The scale bars represent 15 cm.

Fruit firmness measurements were performed in order to ascertain if this important trait had been perturbed (see section 2.1.2). Figure 3-5 shows the fruit firmness measurements which determined over stages of fruit development and ripening.

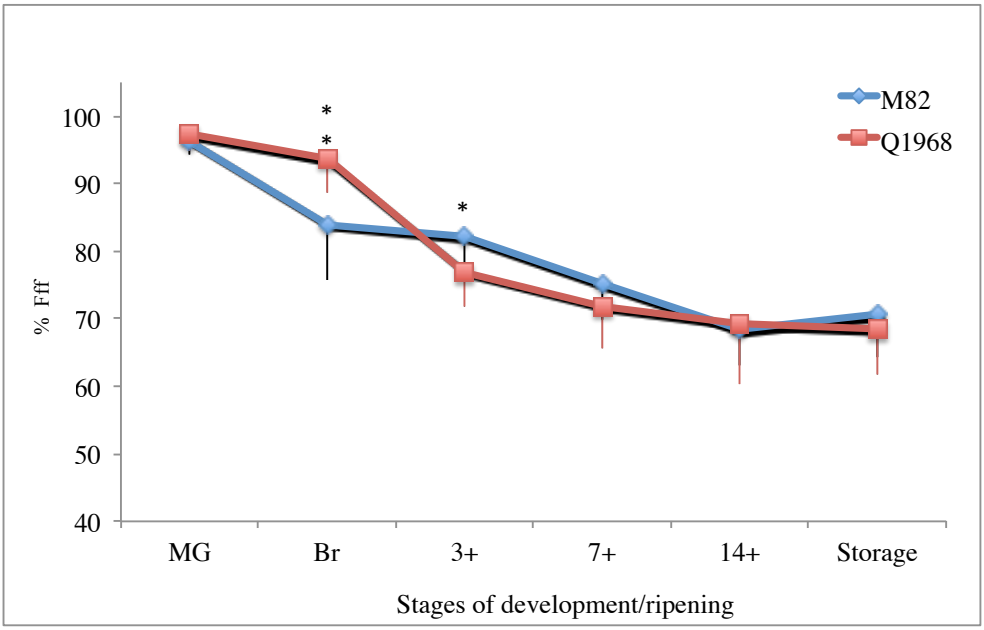



Figure 3-5 A comparison of fruit firmness measurements found in the Q1968 varieties comparison to its M82 comparator. These determinations were made over stages of fruit development and ripening comparing Q1968 lines and control lines (M82).

Red line indicates the percentage of Fruit firmness factor (Fff) in Q1968 lines and blue line shows this amounts in control lines (M82). Measurements were made from three independent biological replicates performed with concurrent technical replicates in triplicate. The ±SD is shown on each column as Error bars. Dunnett's test was used for determination of significant differences between the wild type control lines (M82) and the *S.Pennellii* subIL 2-3/2-4 (Q1968) lines. Significant differences are shown by stars above columns which *, ** and *** are $P<0.05$, $P<0.01$ and $P<0.001$ respectively. MG, +3, +7 and +14 represent Mature green; 3 days post breaker (dpb), 7 dpb and 14 dpb respectively; storage represent 7dpb fruits which stored at 4°C and darkness for 7 more days after harvesting.

The firmness data at the mature green stage showed no difference between Q1968 and M82 lines, but at the breaker stage Q1968 was 10% firmer than the M82 fruits. However by 3 days post breaker (dpb) the trend changed and M86 was firmer compare to Q1968 (6%) (Figure 3-5). In subsequent stages throughout ripening and storage no significant changes in fruit firmness could be observed. As the significant changes in Br and 3 dpb are not considerable, it seems the effects of the introgressed region are not directly associated with fruit softening effects. Further analysis also were performed for better characterisation of Q1968 line in the subsequent sections.

### 3.2.3.2 Analysis of carotenoid pigments and tocopherols in the Q1968 line and its isogenic background (M82) over two seasons and geographical locations

The Q1968 experiments were performed on two batches of fruits which named Crop1 and Crop2. The first batch of samples was grown at Nottingham University (Crop1) and the second batch was grown at RHUL (Crop2). Different experiments were performed on these crops and specially Crop 2 for characterisation of Q1968 line.

Pigment analysis for Crop1 was performed on tomato fruit from the Q1968 line and its M82 comparator. Fruit samples were taken throughout fruit development and ripening the stages were defined as mature green, breaker, 7 days post breaker (7 dpb) and storage. The latter was designated as 7 dpb fruits that stored at 4°C for 7 days. The first batch of samples was grown at Nottingham University under glasshouse conditions with two biological replicates for each stage. A pool of one to three (as provided) fruits was made for each biological replicate and the samples were extracted as described in section 2.2.1.1. Carotenoid extracts (including α-tocopherol) were separated and quantified by UPLC-PDA.

Quantitative and qualitative changes in carotenoid content were observed throughout the developmental and ripening stages analysed. All of the changes are reporting in this thesis are statistically significant changes as judged by their p-values (see section 2.10). Most of the changes occurred at the ripe stage with a decrease in phytoene, phytofluene and lutein (approx. 0.5 fold) but interestingly lycopene increased 1.7 fold to M82 isogenic line. At storage stage an increase of 2.9 and 1.9 fold was detected for lycopene and β-carotene respectively (see Table 3-5) in the Q1968 line. In general differences in carotenoid composition observed in different stages of ripening, and elevation of lycopene in both of ripe and storage stage were considerable result as explained above.

Second Crop of Q1968 and its comparator M82 was grown under glasshouse conditions at RHUL. Based on the first dataset, sample collection was extended to cover more fruit stages over development and ripening. As a result, this staging consisted of mature green, 3 days post breaker (3 dpb), 7 days post breaker (7 dpb), 14 days post breaker (14 dpb) and storage where 7 dpb fruits were stored at 4°C for an additional 7 days. In order to create more robustness in the dataset four biological replicate were collected and analysed. The pool of samples comprised of 2-3 fruits for

each biological replicate, the extraction and pigment detection was subsequently performed in the same manner as that carried out in the first crop. The pigment profile for Q1968 showed changes when compared to the M82 wild type, throughout the developmental and ripening stages analysed (Table 3-6). In mature green, chlorophyll b was not detected in Q1968 line but pheophytin a&b increased 1.3 and 2.4 fold to M82 respectively. α-Tocopherol, β-carotene, and lycopene increased in most of stages. These data can be visualised in Figure 3-6 in order to illustrate the changes in trends arising.

Table 3-5 Crop1 carotenoids and α-tocopherol contents in M82 and Q1968 throughout ripening

Carotenoids and α-tocopherol contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. The samples provided by our colleague in Nottingham University. Samples were made from two independent pools of fruits. Each pool had three technical replicates. Determinations were performed in mature green stage, breaker, 7 days post breaker (ripe) and storage stage (7 days post breaker fruits which stored in 4°C for 7 more days). The mean data are shown with ± SD. Dunnett's test was used to determine significant differences between the background (M82) and Q1968 line. Significant differences are shown in bold. P values with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively.

| | **Stages of fruit development and ripening** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mature Green** | | **Breaker** | | **Ripe** | | **Storage** | |
| | **M82** | **Q19** | **M82** | **Q19** | **M82** | **Q19** | **M82** | **Q19** |
| **α-Tocopherol** | 67 ± 12 | 112 ± 41 | 170 ± 36 | **376 ± 134*** | 212 ± 49 | 246 ± 127 | 353 ± 136 | 497 ± 63 |
| **Chlorophyll b** | 41 ± 17 | 59 ± 16 | 16 ± 5 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| **Pheophytin** | 25 ± 1 | 20 ± 6 | 11 ± 5 | **27 ± 8**** | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| **Phytoene** | 0 ± 0 | 0 ± 0 | 37 ± 27 | 11 ± 1 | 216 ± 38 | **116 ± 42**** | 269 ± 146 | 263 ± 22 |
| **Phytofluene** | 0 ± 0 | 0 ± 0 | 49 ± 2 | 7 ± 0* | 172 ± 50 | **99 ± 38*** | 248 ± 163 | 263 ± 19 |
| **Lycopene** | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 135 ± 72 | **305 ± 101*** | 307 ± 210 | **882 ± 70**** |
| **Lutein** | 23 ± 1 | 20 ± 6 | 22 ± 1 | **48 ± 17*** | 28 ± 8 | **14 ± 4**** | 14 ± 5 | 17 ± 3 |
| **B-Carotene** | 80 ± 6 | 95 ± 29 | 187 ± 96 | **421 ± 85**** | 237 ± 21 | 252 ± 84 | 260 ± 113 | **482 ± 22**** |
| **Total Carotenoids** | 101 ± 7 | 96 ± 54 | 262 ± 140 | **478 ± 97*** | 787 ± 137 | 786 ± 266 | 1098 ± 630 | **1906 ± 69*** |

Table 3-6 Crop2 carotenoids and α-tocopherol contents in M82 and Q1968 throughout ripening.

Carotenoids and α-tocopherol contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Samples were made from three independent pools of fruits. Each pool had four technical replicates. Determinations were performed in mature green stage, 3, 7 and 14 days post breaker and storage stage (7 days post breaker fruits which stored in 4°C for 7 more days). The mean data are shown with ± SD. Dunnett's test was used to determine significant differences between the background (M82) and Q1968 line. Significant differences are shown in bold. P values with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively.

| | **Stages of fruit development and ripening** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Mature Green** | | **+3** | | **+7** | | **+14** | | **Storage** | |
| | **M82** | **Q19** | **M82** | **Q19** | **M82** | **Q19** | **M82** | **Q19** | **M82** | **Q19** |
| **α-Tocopherol** | 61 ± 25 | 71 ± 29 | 149 ± 24 | **215 ± 60**** | 164 ± 59 | **222 ± 52*** | 142 ± 50 | 131 ± 32 | 168 ± 39 | **208 ± 36*** |
| **Chlorophyll b** | 99 ± 35 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| **Pheophytin a** | 18 ± 4 | **23 ± 3**** | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| **Pheophytin b** | 31 ± 20 | **75 ± 9***** | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| **Phytoene** | 0 ± 0 | 0 ± 0 | 74 ± 36 | 78 ± 34 | 234 ± 87 | 192 ± 88 | 343 ± 102 | **471 ± 127*** | 263 ± 73 | **387 ± 40***** |
| **Phytofluene** | 0 ± 0 | 0 ± 0 | 40 ± 20 | 47 ± 9 | 133 ± 78 | 108 ± 72 | 251 ± 77 | 297 ± 63 | 183 ± 49 | 175 ± 106 |
| **Lycopene** | 0 ± 0 | 0 ± 0 | 113 ± 11 | **551 ± 97***** | 664 ± 129 | **1199 ± 288***** | 1362 ± 178 | **1833 ± 156***** | 769 ± 184 | **1320 ± 261***** |
| **Lutein** | 23 ± 4 | 22 ± 6 | 22 ± 5 | 22 ± 3 | 21 ± 8 | **15 ± 3*** | 14 ± 4 | 17 ± 5 | 13 ± 4 | 15 ± 4 |
| **β-carotene** | 0 ± 0 | 0 ± 0 | 31 ± 2 | **56 ± 23**** | 34 ± 6 | **57 ± 9***** | 33 ± 7 | **45 ± 7***** | 33 ± 6 | **47 ± 11**** |
| **Total Carotenoids** | 23 ± 4 | 22 ± 6 | 252 ± 61 | **730 ± 124***** | 919 ± 211 | **1570 ± 410***** | 1982 ± 289 | **2663 ± 257***** | 1246 ± 323 | **1945 ± 374***** |

Differences in lycopene content of Q1968 started with 4.9 fold elevation at 3 dpb and the increase continued over subsequent stages of ripening and storage stage in comparison to M82 control but the level of the increase was not as dramatic approx. 2-fold. β-carotene content also increased 1.8, 1.7 fold for 3 dpb, and 7 respectively and reached to approximately 1.4 fold in 14 dpb and storage stage. α-Tocopherol content of Q1968 was 1.4 times higher in the 3 dpb and 7 dpb fruits. No significant difference was observed for α-Tocopherol at 14 dpb, but in the storage stage the changes were increased 1.2 fold for the Q1968 line in comparison to M82 (Figure 3-6). In general, the amount of total carotenoids was higher in all of stages of ripening in Q1968 line except for the mature green stage, where there was no detectable change. Lycopene represented the greatest perturbation.

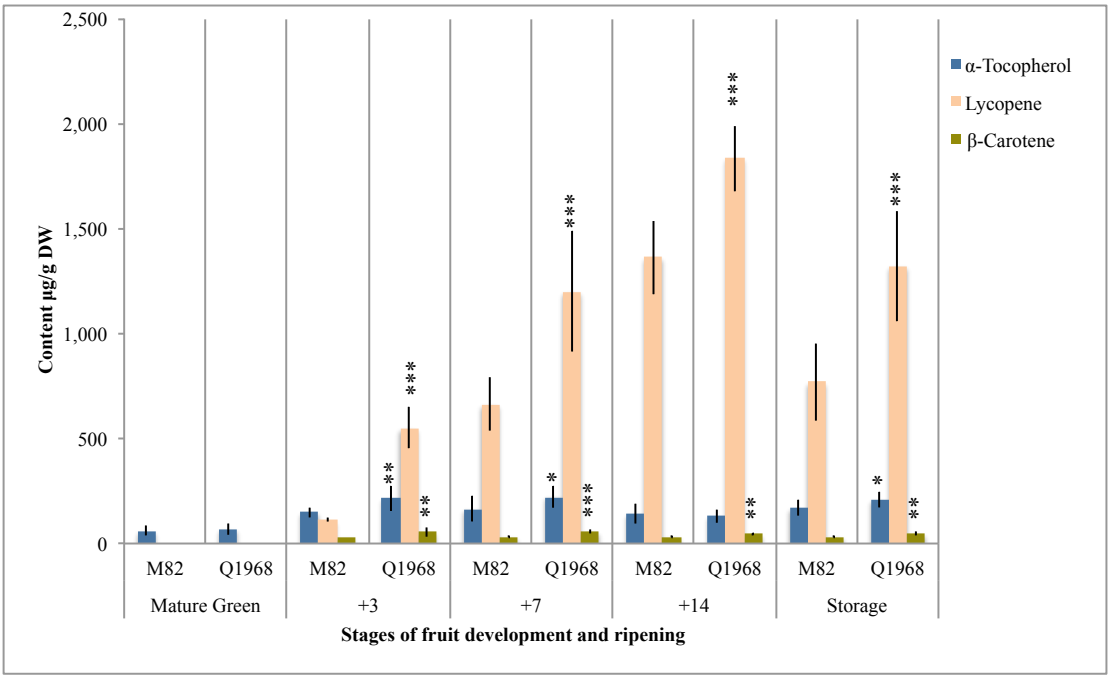



Figure 3-6 Some carotenoids and α-Tocopherol contents in M82 and Q1968 throughout ripening with RHUL grown samples.

Carotenoids and α-tocopherol contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Samples were made from three independent pools of fruits. Each pool had four technical replicates. Determinations were performed in mature green stage, 3, 7 and 14 days post breaker and storage stage (7 days post breaker fruits which stored in 4°C for 7 more days). Error bars indicate ± SD. Dunnett's test was used to determine significant differences between the background (M82) and Q1968 line. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

#### 3.2.3.3 Metabolite profile changes during fruit development and ripening in Q1968 line

For a better understanding about other metabolite variation between Q1968 and M82 comparator lines, metabolite profiling was performed by GC MS and Statistical analyses were carried out for quantification of metabolites as described in section 2.2.4. Over 60 metabolites identified and quantified in 5 stages of development and ripening in Q1968 line and compared to its wild type M82 control line. Isoprenoids level alterations have been described in section 3.2.3.2. Most of changes in metabolites level of Q1968 were observed in 7 dpb, 14 dpb and storage stage while MG and 3 dpb did not show a great difference with M82 wild type. Some sugars decreased in 7 dpb stage in Q1968 line but in rest of stages no difference was detected. Most of changes in organic acids levels occurred in 7 dpb and 14 dpb time points. Considerable changes of 5 and 9-fold increase were occurred in ascorbic acid content of Q1968 line to wild type M82 in 7 dpb and 14 dpb respectively. Amino acids category represented the most alterations in all of stages in comparison to other classes. Nine amino acids of Q1968 in 14 dpb stage elevated 1.7 to 5 fold to M82 and in storage stage 8 of them increased 2 to 4 fold. In general Q1968 mostly make the differences in 7 dpb, 14dpb and storage steps of ripening and the most varied class of compound was amino acids (see Table 3-7).

Table 3-7 The ratio of metabolite changes at different stages of fruit development and ripening in the Q1968 line tomato fruits compared to M82 wild type

Data obtained from GC-MS analytical platform with subsequent statistical analysis. The data are presented as mean ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively. If a metabolite is unique to Q1968 at the concentration used is shown as In Inc (indefinite increase); and when is unique to M82 at the sample concentration used is shown as In Dec (indefinite decrease) and "-" Indicates metabolite was not detectable in both Q1968 and M82 at the sample concentration used. GABA: γ-Aminobutyric acid.

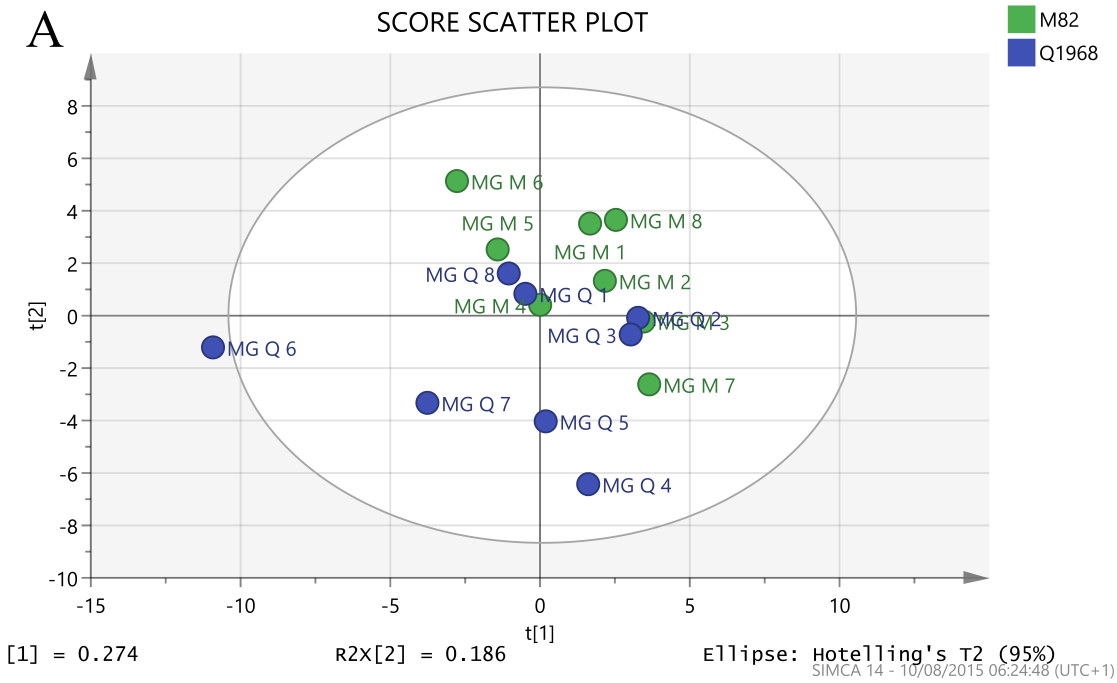
| | Q1968/M82 Ratio | | | | |
|---|---|---|---|---|---|
| **Metabolite** | **MG** | **+3** | **+7** | **+14** | **S** |
| **Amino Acid** | | | | | |
| Alanine | 1.63 ± 1.09 | - | 1.47 ± 0.79 | **5.47 ± 2.38** ** | 2.46 ± 1.22 |
| Asparagine | - | 1.35 ± 2.14 | - | - | In Inc |
| beta-Alanine | 3.14 ± 2.82 | **2.62 ± 1.65** * | **1.87 ± 0.71** * | **5.11 ± 2.79** * | **3.95 ± 1.51** *** |
| Cysteine | - | - | **In Dec** | **2.13 ± 0.79** * | 0.78 ± 0.46 |
| GABA | 1.59 ± 1.2 | 1.42 ± 0.74 | 1.53 ± 0.44 | **2.35 ± 1.37** * | **3.33 ± 1.45** ** |
| Dihydrouracil | 1.78 ± 1.28 | 1.45 ± 1.01 | 2.12 ± 1.27 | **4.57 ± 2.99** * | **2.44 ± 1.16** * |
| Glutamine | 2.62 ± 3.55 | 1.79 ± 1.15 | 1.84 ± 1.11 | **4.94 ± 3.41** * | **2.02 ± 0.59** ** |
| Glycine | 6.31 ± 6.08 | **In Inc** | 2.07 ± 1.19 | **5.07 ± 4.48** * | **3.47 ± 1.61** ** |
| Homocysteine | - | - | **1.3 ± 0.3** * | **In Dec** | - |
| Isoleucine | 1.77 ± 0.43 | 5.1 ± 4.07 | **2.24 ± 1.06** * | **In Inc** | **2.74 ± 0.73** ** |
| Leucine | 1.49 ± 0.98 | **2.86 ± 1.63** * | 1.47 ± 0.49 | **4.51 ± 2.4** ** | 1.45 ± 0.63 |
| Lysine | **3.98 ± 2.21** ** | **1.9 ± 0.51** ** | 1.11 ± 0.74 | **2.12 ± 1.02** * | 1.45 ± 0.67 |
| Methionine | 2.21 ± 1.83 | 2.79 ± 1.9 | 1.21 ± 0.36 | 3.63 ± 3.1 | 1.28 ± 0.76 |
| Phenylalanine | 0.91 ± 0.3 | 1.83 ± 1.04 | 2.56 ± 1.93 | 3.02 ± 2.26 | **3.77 ± 2.97** * |
| Putrescine | 0.4 ± 0.08 | 1.47 ± 0.44 | - | **1.7 ± 0.41** * | 2.33 ± 1.55 |
| Serine | 1.5 ± 0.94 | **2 ± 0.93** * | 1.24 ± 0.28 | **2.04 ± 0.83** * | **2.3 ± 1.06** * |
| Threonine | 1.57 ± 0.85 | 1.99 ± 1.11 | 1.28 ± 0.31 | 1.49 ± 0.69 | 1.76 ± 0.8 |
| Tryptophan | - | - | **In Dec** | **In Dec** | 0.94 ± 0.29 |
| Valine | 0.98 ± 0.59 | 4.32 ± 3.47 | 1.04 ± 0.25 | 3.64 ± 3.32 | **2.85 ± 2.02** * |
| **Lipid** | | | | | |
| Glycero-1-C16:0 | 0.9 ± 0.29 | 2.04 ± 1.66 | **1.7 ± 0.59** * | 1.32 ± 0.26 | 1.09 ± 0.4 |
| Glycero-1-C18:0 | 2.32 ± 1.08 | 1.53 ± 1.33 | 1.6 ± 0.7 | **4.93 ± 3.26** * | 2.17 ± 1.34 |
| Mesaconic acid | 1.25 ± 0.4 | 1.36 ± 0.65 | **0.7 ± 0.12** *** | **0.67 ± 0.16** ** | 0.92 ± 0.25 |
| Palmitic acid | 0.79 ± 0.24 | 1.73 ± 0.95 | 0.98 ± 0.13 | 0.73 ± 0.32 | 0.95 ± 0.47 |
| Stearic acid | 0.59 ± 0.19 | 4.09 ± 3.86 | 1 ± 0.38 | **In Dec** | 1.23 ± 1.16 |
| **Organic Acid** | | | | | |
| 2-ketoglutaric acid | 1.62 ± 0.99 | 1 ± 0.46 | **In Dec** | 1.26 ± 0.68 | 1.08 ± 0.41 |
| Aconitic acid | **0.64 ± 0.37** * | 0.72 ± 0.21 | **0.54 ± 0.17** * | 1.13 ± 0.5 | 1.22 ± 0.36 |
| Ascorbic acid | - | 1.79 ± 0.97 | **4.94 ± 1.97** *** | **9.06 ± 3.11** ** | 3.99 ± 2.32 |
| Aspartic acid | 0.85 ± 0.39 | 0.89 ± 0.72 | 1.84 ± 1.37 | **4.09 ± 2.9** * | 2.25 ± 1.44 |

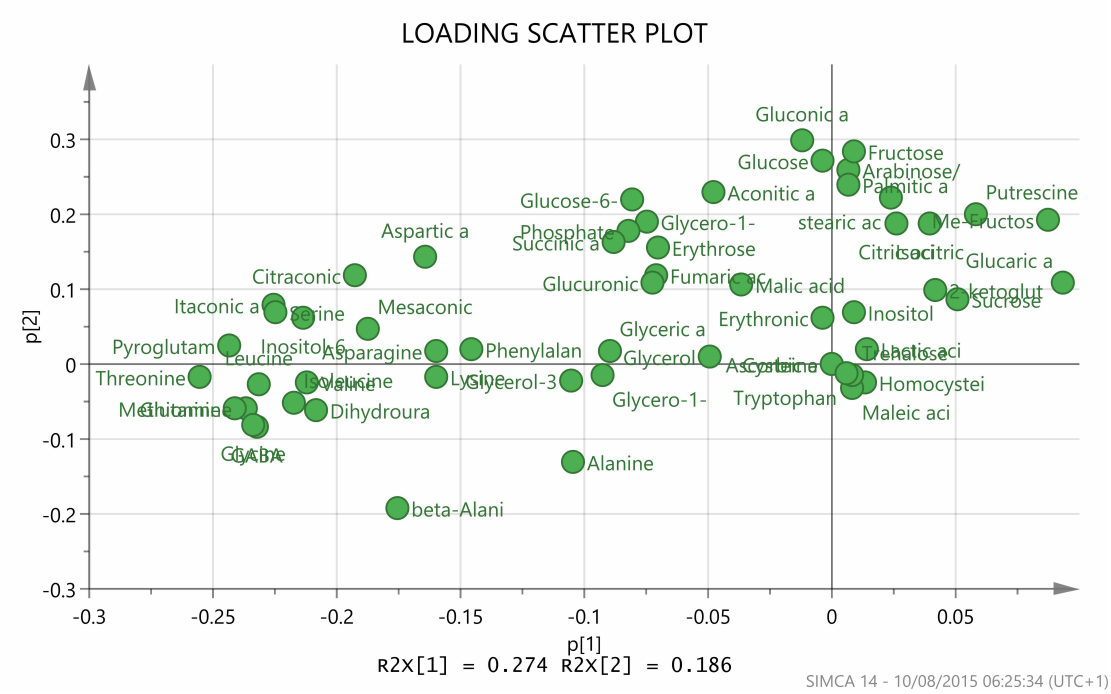
| | | | | | |
|---|---|---|---|---|---|
| Citraconic acid | 0.96 ± 0.24 | 1.21 ± 0.34 | **0.7 ± 0.08 *** ** | 0.86 ± 0.15 | 1.01 ± 0.21 |
| Citric acid | **0.22 ± 0.21 ** ** | **0.51 ± 0.28 ** ** | 0.59 ± 0.39 | 1.09 ± 0.58 | 1.1 ± 0.68 |
| Erythronic acid | 1.82 ± 1.14 | 1.13 ± 0.42 | 2.21 ± 1.54 | **1.65 ± 0.59 *** | 1.19 ± 0.29 |
| Fumaric acid | 1.02 ± 0.27 | 1.39 ± 0.66 | 0.91 ± 0.43 | **0.64 ± 0.13 ** ** | 1.12 ± 0.41 |
| Glucaric acid | **0.12 ± 0.07 *** | **0.29 ± 0.21 *** | 1.4 ± 0.61 | 1.86 ± 0.61 | 1.07 ± 0.47 |
| Gluconic acid | **0.7 ± 0.23 *** | 1.02 ± 0.35 | **0.6 ± 0.2 ** ** | 1.01 ± 0.41 | 1.13 ± 0.38 |
| Glucuronic acid | 0.95 ± 0.35 | 1.33 ± 0.41 | 1.11 ± 0.28 | 0.91 ± 0.46 | 1.2 ± 0.34 |
| Glyceric acid | **2.67 ± 1.63 *** | 1.29 ± 0.77 | **1.81 ± 0.73 *** | **1.61 ± 0.53 *** | 1.51 ± 0.68 |
| Itaconic acid | 1.02 ± 0.28 | 1.37 ± 0.56 | **0.63 ± 0.1 *** ** | **0.72 ± 0.16 *** | 0.78 ± 0.18 |
| Lactic acid | 0.71 ± 0.15 | 0.8 ± 0.27 | 0.91 ± 0.38 | 0.48 ± 0.27 | 0.51 ± 0.24 |
| Maleic acid | 1.22 ± 0.35 | 1.41 ± 0.97 | - | **In Dec** | **In Dec** |
| Malic acid | 0.84 ± 0.52 | 0.79 ± 0.25 | 0.93 ± 0.57 | 1.12 ± 0.27 | 0.84 ± 0.32 |
| Pyroglutamic acid | 1.1 ± 0.6 | 1.42 ± 1.09 | 1.3 ± 0.95 | 2.37 ± 1.65 | **2.47 ± 0.99 ** ** |
| Succinic acid | **0.68 ± 0.17 *** | 1.07 ± 0.33 | 1.99 ± 1.35 | **2.58 ± 1.21 *** | **1.65 ± 0.28 *** ** |
| **Phosphate** | | | | | |
| Glucose-6-Phosphate | 0.85 ± 0.4 | 0.87 ± 0.35 | 0.48 ± 0.41 | 1.03 ± 0.57 | **2.08 ± 0.75 ** ** |
| Glycerol-3-Phosphate | 1.26 ± 0.66 | 1.67 ± 1.28 | 1.74 ± 0.65 | 1.53 ± 0.44 | 1.54 ± 0.52 |
| Inositol-6-Phosphate | 0.97 ± 0.61 | 1.28 ± 0.38 | **2.74 ± 1.54 *** | **1.77 ± 0.47 ** ** | **2.23 ± 0.89 *** |
| Phosphate | 0.57 ± 0.17 | 0.92 ± 0.46 | 1.13 ± 0.42 | **1.42 ± 0.37 *** | 1.38 ± 0.79 |
| **Polyol** | | | | | |
| Glycerol | **2.13 ± 1.17 *** | 2 ± 1.36 | **1.68 ± 0.61 *** | 0.96 ± 0.32 | 2.94 ± 2.4 |
| Inositol | 0.87 ± 0.16 | 1.35 ± 0.59 | 0.77 ± 0.28 | 1.05 ± 0.51 | 1.09 ± 0.39 |
| **Sugar** | | | | | |
| Arabinose/Ribose/ Xylose | 0.88 ± 0.28 | 0.99 ± 0.23 | **0.47 ± 0.35 *** | 0.63 ± 0.27 | 0.89 ± 0.35 |
| Erythrose | 1.13 ± 0.75 | 1.75 ± 0.8 | **In Dec** | 1.74 ± 0.98 | 1.74 ± 0.47 |
| Fructose | 0.82 ± 0.23 | 1.01 ± 0.21 | 0.9 ± 0.33 | 1 ± 0.31 | 1.17 ± 0.23 |
| Glucose | 0.85 ± 0.26 | 0.97 ± 0.35 | **0.74 ± 0.23 *** | 0.75 ± 0.24 | 0.93 ± 0.25 |
| Maltose | - | 1.04 ± 0.34 | **In Dec** | 1.47 ± 0.88 | 2.18 ± 1.37 |
| Me-Fructose | 0.54 ± 0.45 | **2.12 ± 1.06 *** | 1.28 ± 0.47 | 0.99 ± 0.38 | 0.98 ± 0.26 |
| Sucrose | 0.62 ± 0.47 | **In Dec** | **In Dec** | **In Dec** | - |
| Trehalose | - | 1.72 ± 0.85 | **0.55 ± 0.35 *** | 1.29 ± 0.69 | 1.42 ± 0.42 |

Multivariate principal component analysis (PCA) was performed and presented as score scatter plots illustrating the overall variance of chemical composition between M82 and Q1968 lines, and loading scatter plots representing contribution of each metabolite to the overall variance. PCA was performed for 5 different stages of fruit ripening in M82 and Q1968 (see Figure 3-7).

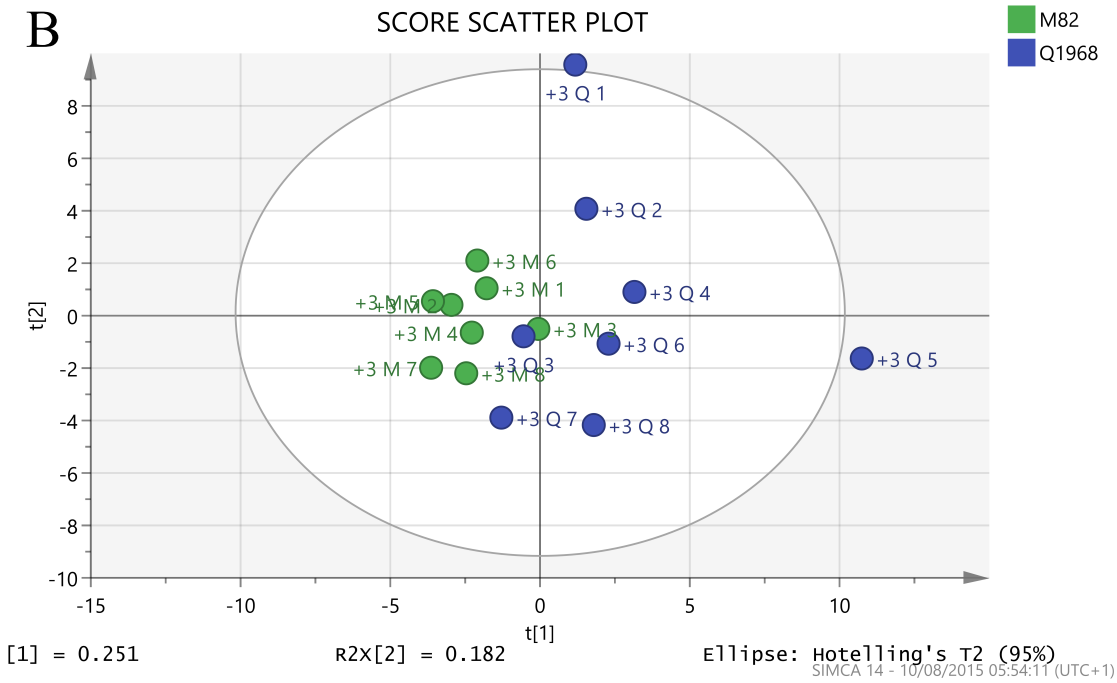
In mature green stage the score value of scatter plot did not show a significant different cluster for M82 and Q1968. In the loading scatter plot some shift of metabolites to left panel was observed which was a result of variability in a Q1968

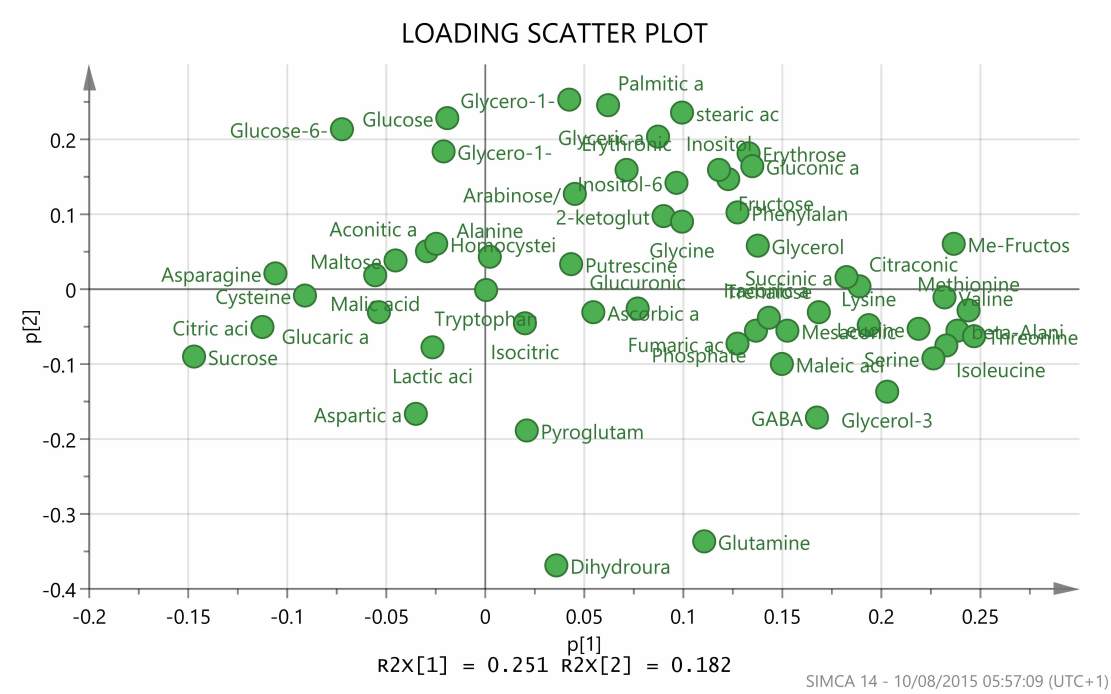
sample, resulting in a shift to the left plane (Figure 3-7 A). From 3 days post breaker the separation of M82 and Q1968 samples were more pronounced in the score scatter plots. In this stage most of M82 samples clustered together in the left side and Q1968 lines were more spread in the right hand side of the plot. The loading plot of this stage indicated some amino acids like threonine, valine, beta alanine, serine, isoleucine and methionine had significant weightings and the highest loading in Q1968 (Figure 3-7 B). In 7 dpb stage the Q1968 and M82 clearly separated both in the score scatter plot and in the loading plot some amino acids (beta alanine, glutamine, aspartic acid, etc.) and some organic acids (ascorbic acid, succinic acid, etc.) grouped together on left hand side suggesting a specific chemical composition affect on Q1968 at this stage (Figure 3-7 C). In 14 days and storage stages Q1968 samples and M82 samples grouped separately from each other due to their score values. The loading scatter plots at 14 dpb a group of highly weighted chemical compounds (mostly amino acids) affected Q1968 but no specific clustering of compounds was observed for M82 at any of stages. Metabolites were distributed evenly on the scatter plot s generated for the loading values associated with storage, suggesting their influence was similar on both M82 and Q1968.


A
SCORE SCATTER PLOT
M82
Q1968
MG M 6
MG M 5
MG M 8
MG M 1
MG Q 8
MG M 2
MG Q 1
MG M 4
MG Q 2
MG M 3
MG Q 3
MG Q 6
MG M 7
MG Q 7
MG Q 5
MG Q 4
t[2]
t[1]
[1] = 0.274
R2X[2] = 0.186
Ellipse: Hotelling's T2 (95%)
SIMCA 14 - 10/08/2015 06:24:48 (UTC+1)

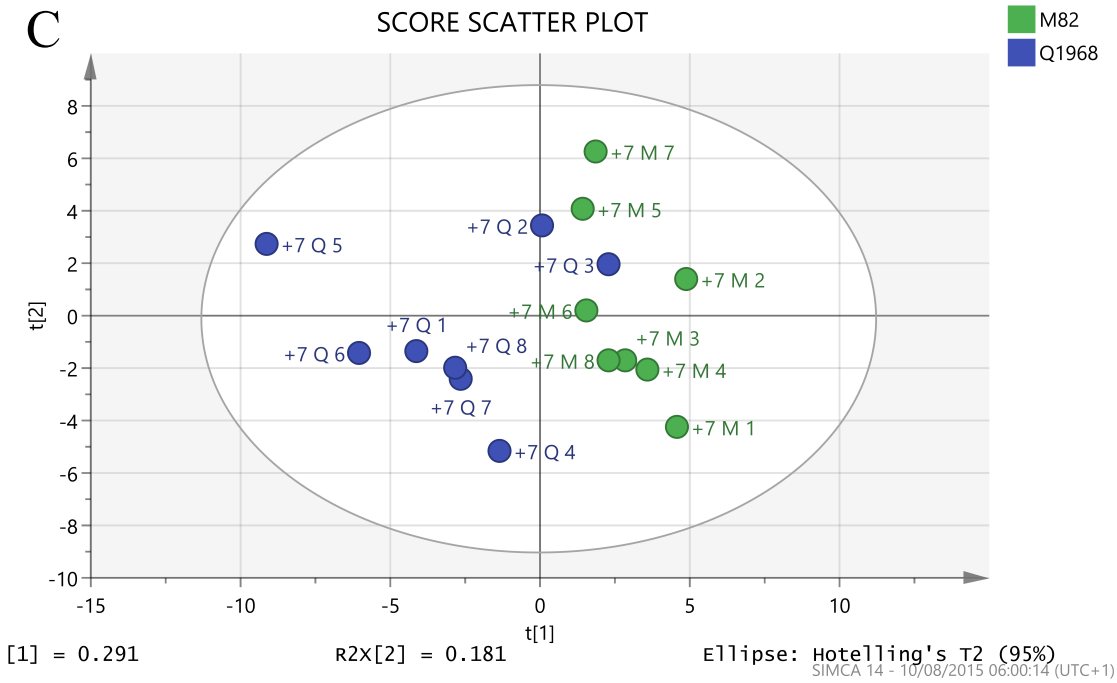

LOADING SCATTER PLOT
Gluconic a
Fructose
Glucose
Arabinose/
Palmitic a
Putrescine
Glucose-6-
Aconitic a
Glycero-1-
stearic ac
Aspartic a
Phosphate
Succinic a
Erythrose
Citraconic
Glucuronic
Malic acid
Glucaric a
Itaconic a
Mesaconic
Erythronic
Inositol
Sucrose
Glyceric a
Pyroglutam
Asparagine
Phenylalan
Glycerol
Threonine
Leucine
Glycerol-3
Homocystei
Glycero-1-
Tryptophan
Maleic aci
Dihydroura
Alanine
beta-Alani
p[2]
p[1]
R2X[1] = 0.274 R2X[2] = 0.186
SIMCA 14 - 10/08/2015 06:25:34 (UTC+1)

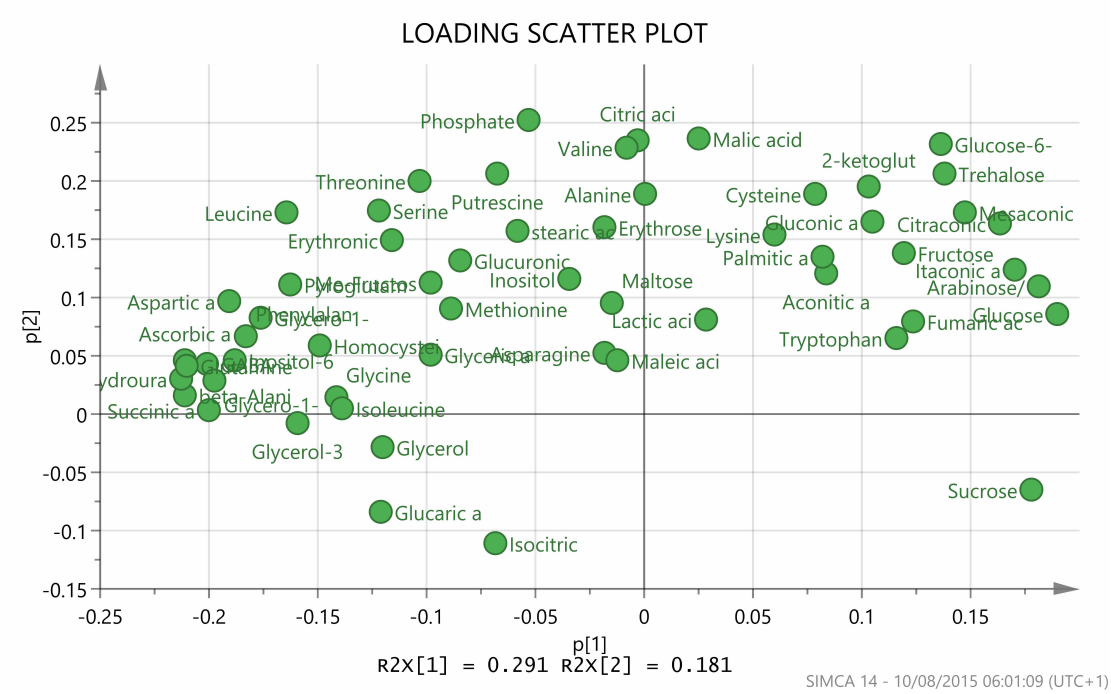
B


SCORE SCATTER PLOT
M82
Q1968
+3 Q 1
+3 Q 2
+3 M 6
+3 M 1
+3 Q 4
+3 M 4
+3 M 3
+3 Q 6
+3 Q 5
+3 M 7
+3 Q 3
+3 M 8
+3 Q 7
+3 Q 8
t[2]
t[1]
[1] = 0.251
R2X[2] = 0.182
Ellipse: Hotelling's T2 (95%)
SIMCA 14 - 10/08/2015 05:54:11 (UTC+1)

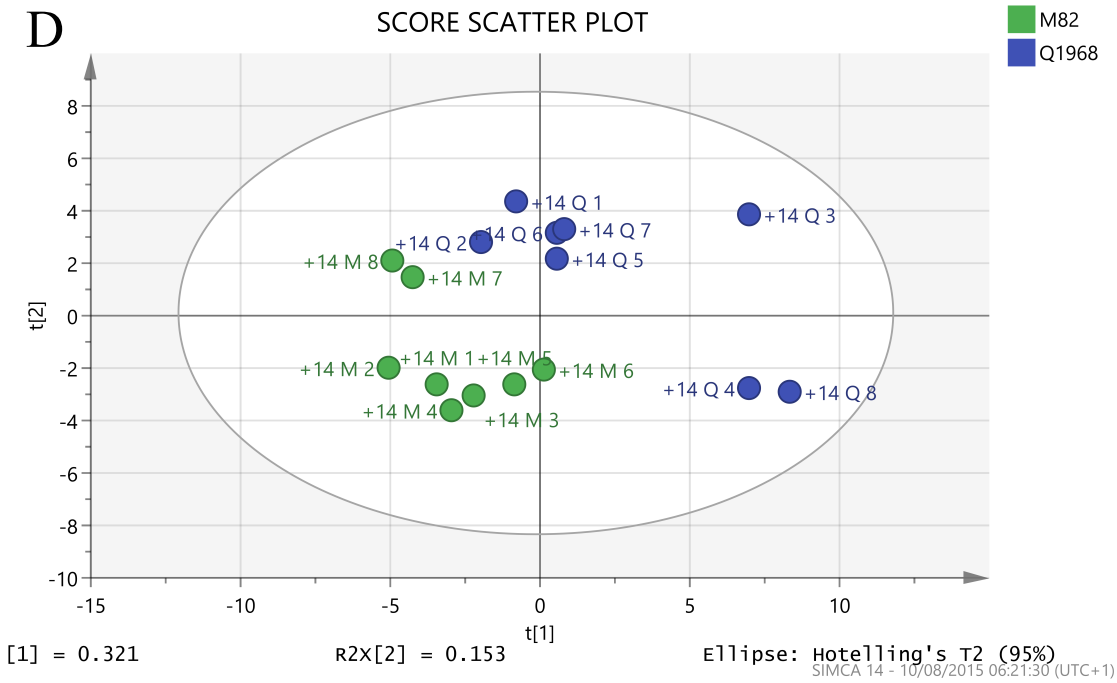

LOADING SCATTER PLOT
Palmitic a
Glycero-1-
Glucose
Glucose-6-
stearic ac
Glycero-1-
Inositol
Erythrose
Gluconic a
Inositol-6
Arabinose/
Fructose
2-ketoglut
Phenylalan
Aconitic a
Alanine
Glycine
Glycerol
Me-Fructos
Asparagine
Maltose
Putrescine
Citraconic
Glucuronic
Succinic a
Methionine
Cysteine
Lysine
Valine
Malic acid
Tryptophan
Ascorbic a
Citric aci
Glucaric a
Fumaric ac
Beta-Alani
Threonine
Sucrose
Isocitric
Phosphate
Maleic aci
Serine
Isoleucine
Lactic aci
Aspartic a
GABA
Glycerol-3
Pyroglutam
Glutamine
Dihydroura
p[2]
p[1]
R2X[1] = 0.251 R2X[2] = 0.182
SIMCA 14 - 10/08/2015 05:57:09 (UTC+1)

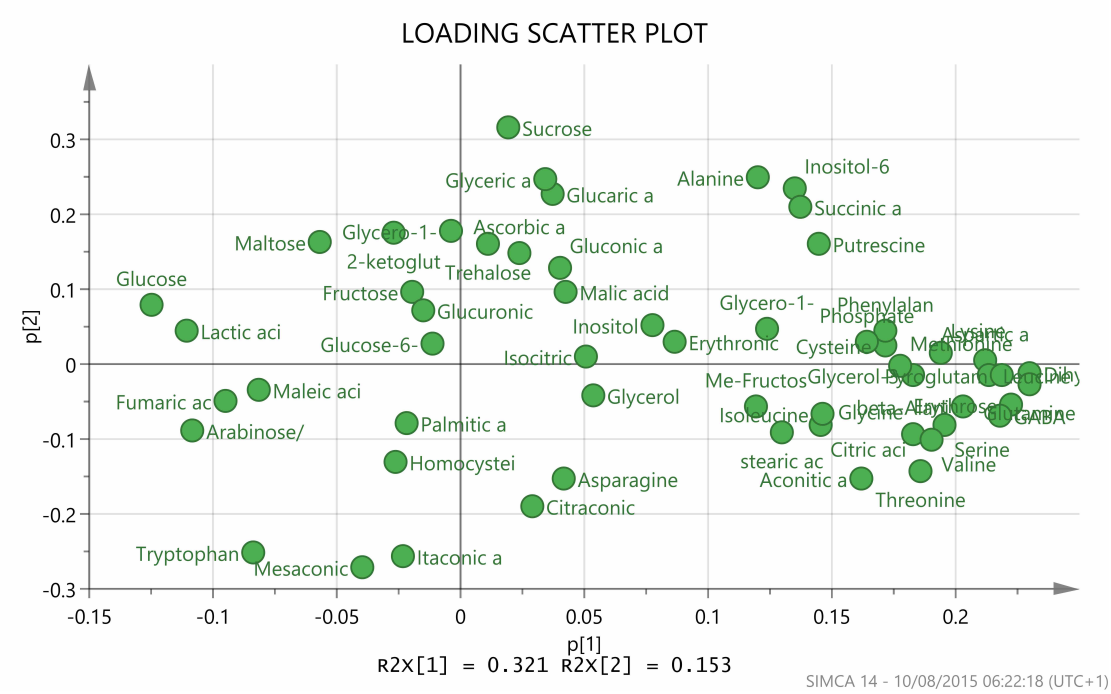
C


SCORE SCATTER PLOT
M82
Q1968
+7 M 7
+7 M 5
+7 Q 2
+7 Q 5
+7 Q 3
+7 M 2
+7 M 6
+7 Q 1
+7 M 3
+7 Q 6
+7 Q 8
+7 M 8
+7 M 4
+7 Q 7
+7 M 1
+7 Q 4
t[2]
t[1]
[1] = 0.291
R2X[2] = 0.181
Ellipse: Hotelling's T2 (95%)
SIMCA 14 - 10/08/2015 06:00:14 (UTC+1)


LOADING SCATTER PLOT
Phosphate
Citric aci
Malic acid
Valine
Glucose-6-
2-ketoglut
Trehalose
Threonine
Putrescine
Alanine
Cysteine
Leucine
Serine
Mesaconic
Gluconic a
Citraconic
Erythronic
Erythrose
Lysine
Glucuronic
Palmitic a
Fructose
Inositol
Maltose
Itaconic a
Arabinose/
Aspartic a
Methionine
Aconitic a
Lactic aci
Glucose
Ascorbic a
Fumaric ac
Tryptophan
Homocystei
Asparagine
Maleic aci
Glycine
Isoleucine
Glycerol-3
Glycerol
Sucrose
Glucaric a
Isocitric
p[2]
p[1]
R2X[1] = 0.291 R2X[2] = 0.181
SIMCA 14 - 10/08/2015 06:01:09 (UTC+1)

D


SCORE SCATTER PLOT
M82
Q1968
t[2]
t[1]
+14 Q 1
+14 Q 3
+14 Q 7
+14 Q 2
+14 Q 6
+14 Q 5
+14 M 8
+14 M 7
+14 M 2
+14 M 1
+14 M 5
+14 M 6
+14 Q 4
+14 Q 8
+14 M 4
+14 M 3
[1] = 0.321
R2X[2] = 0.153
Ellipse: Hotelling's T2 (95%)
SIMCA 14 - 10/08/2015 06:21:30 (UTC+1)


LOADING SCATTER PLOT
p[2]
p[1]
Sucrose
Glyceric a
Glucaric a
Alanine
Inositol-6
Succinic a
Maltose
Glycero-1-
Ascorbic a
Gluconic a
Putrescine
2-ketoglut
Trehalose
Glucose
Fructose
Malic acid
Glucuronic
Glycero-1-
Phenylalan
Phosphate
Inositol
Lactic aci
Glucose-6-
Erythronic
Cysteine
Isocitric
Fumaric ac
Maleic aci
Glycerol
Arabinose/
Palmitic a
Isoleucine
Citric aci
Serine
stearic ac
Homocystei
Valine
Asparagine
Aconitic a
Citraconic
Threonine
Tryptophan
Mesaconic
Itaconic a
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SIMCA 14 - 10/08/2015 06:22:18 (UTC+1)

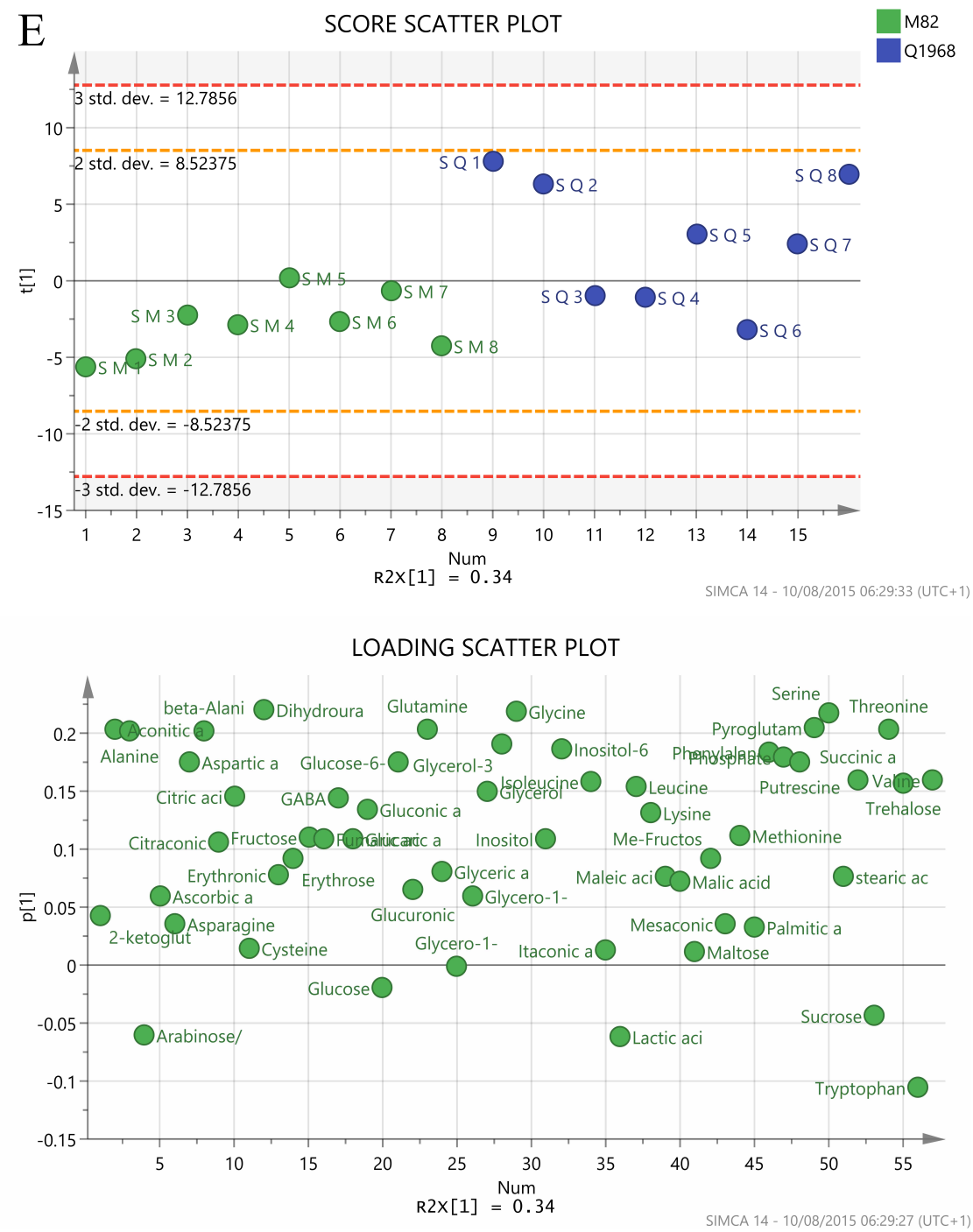
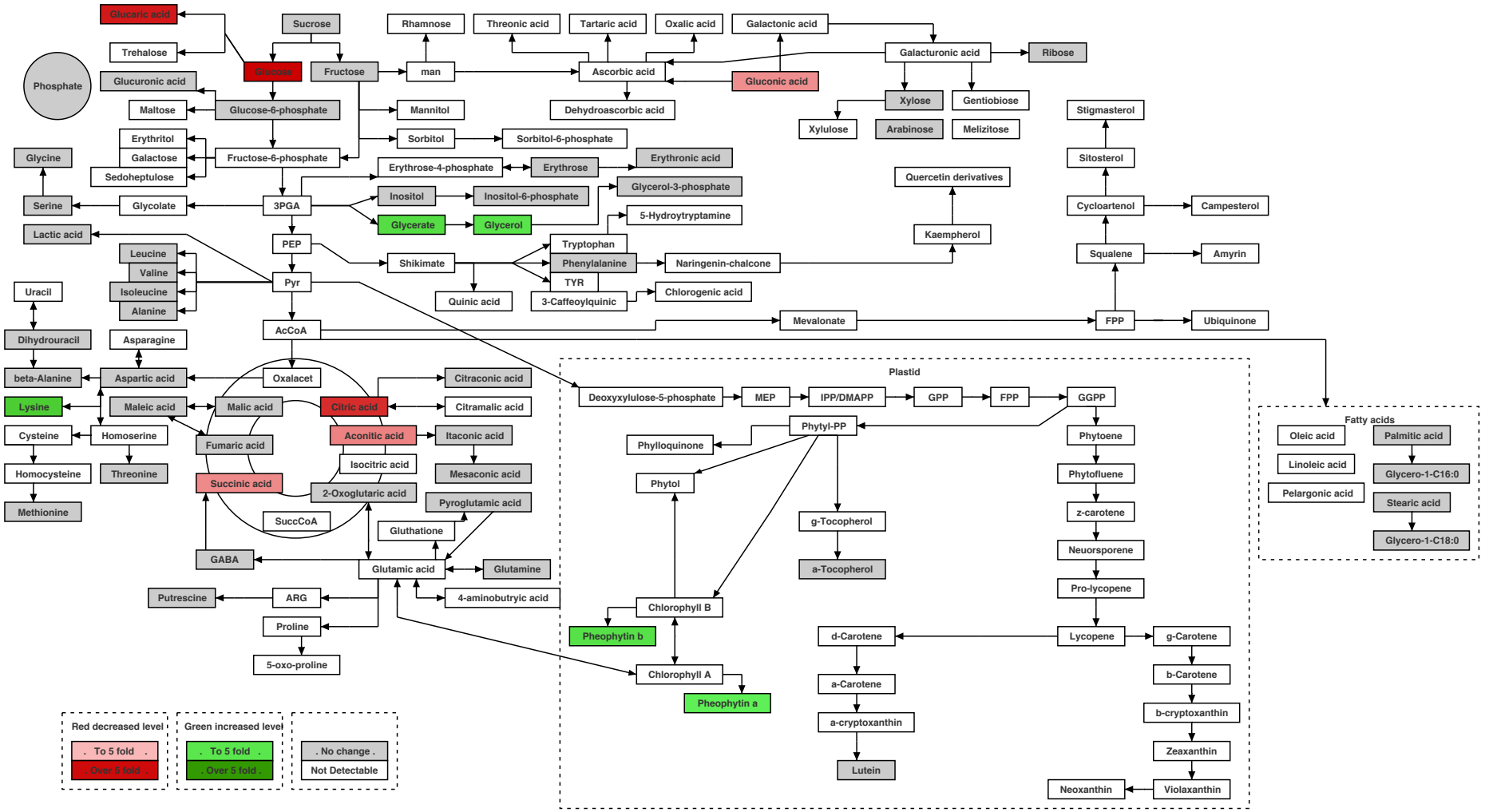



Figure 3-7 Principal component analysis (PCA) of M82 (control lines) and Q1968 tomato fruits at 5 different stages of ripening. The metabolites represented were detected by GC-MS.

Score scatter plots and loading scatter plots are illustrated for (A) Mature green stage, (B) 3 days post breaker, (C) 7 days post breaker, (D) 14 days post breaker, (E) Storage stage (7 days post breaker fruits stored at 4°C for 7 more days). These subsequent stages of ripening are shown as MG, +3, +7, +14 and S respectively in score scatter plots. Also in score scatter plots numbers indicate the sample numbers and M and Q represent M82 and Q1968 respectively. The full names of the variables (metabolites) on the loading scatter plots are available in Table 3-7. Four biological with minimum two technical replicates were analysed. The extraction method, analytical and data processing method used to analyse polar metabolites are described in sections 2.2.4.

For a better visual comparison about the metabolite alterations in fruits at each stage, the changes were represented in biochemical pathways (Figure 3-8). The changes are illustrated as colour change reflecting the fold changes of metabolite ratio in Q1968 to M82. These visual figures help for a better understanding of the overall changes occurring at different stages of ripening. As the ripening stages went further from MG to 14 dpb (Figure 3-8 A-D) the number of quantitative metabolite changes increased. These changes were mostly increases in some amino acids, and carotenoids. At the 14 dpb stage an elevation in some of organic acids was notable. Storage and the 14 dpb stage were equal in days of ripening after breaker; with this difference that the last 7 days for storage samples were spent in dark at 4°C while 14 dpb samples remained attached to their plants. During the storage conditions (Figure 3-8 E) some changes to the 14 dpb time point were visible for instance the level of ascorbic acid in Q1968 was not significantly higher to M82 during storage. The types of amino acids changed were also different at this step.

A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Gluconic acid
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Galactose
Fructose-6-phosphate
Erythrose-4-phosphate
Erythrose
Erythronic acid
Sitosterol
Sedoheptulose
Quercetin derivatives
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Glycerol-3-phosphate
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3PGA
Cycloartenol
Campesterol
Glycerate
Glycerol
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Tryptophan
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FPP
GGPP
beta-Alanine
Aspartic acid
Oxalacet
Citraconic acid
Fatty acids
Lysine
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Malic acid
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Citramalic acid
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Oleic acid
Palmitic acid
Cysteine
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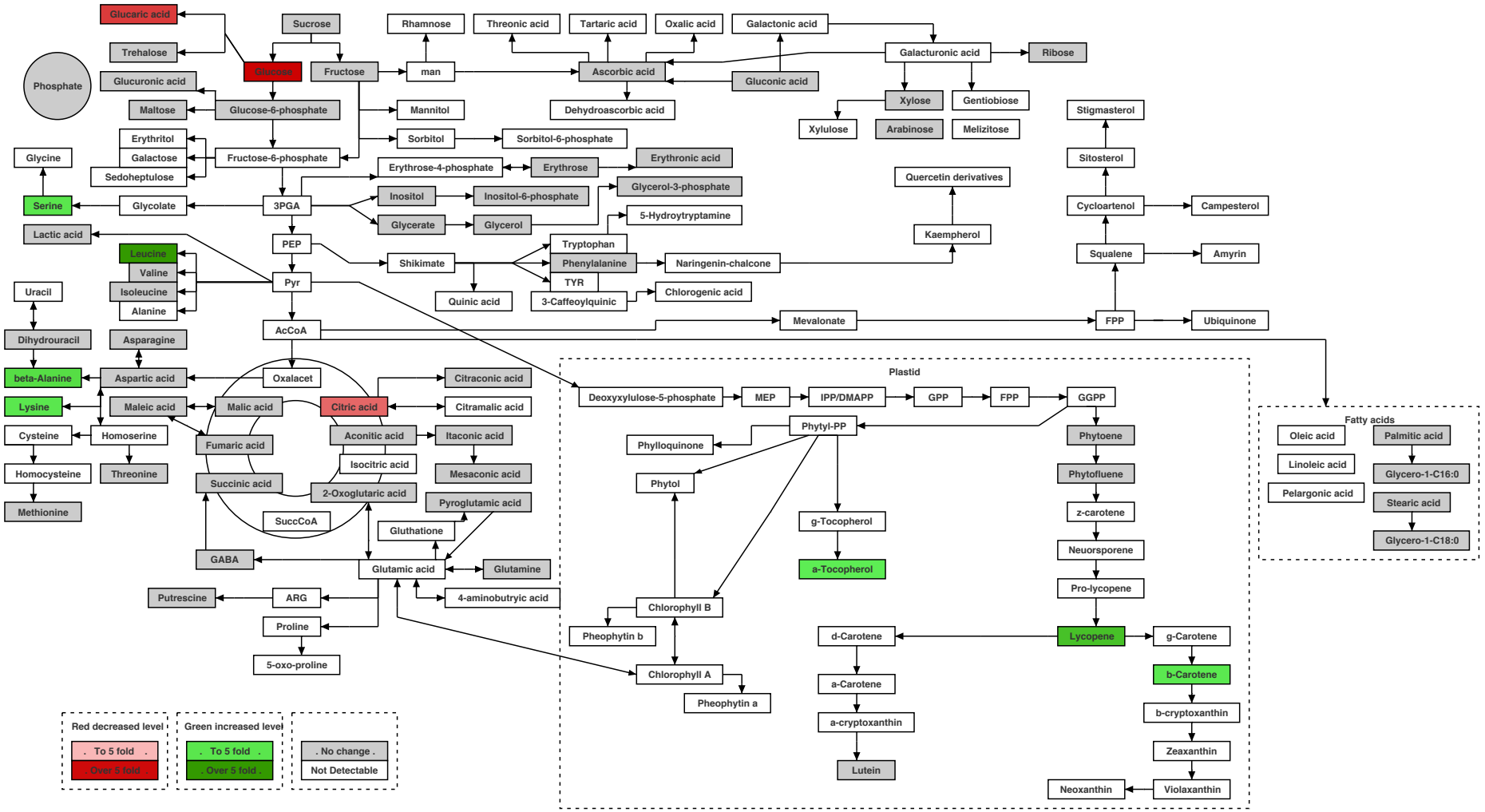
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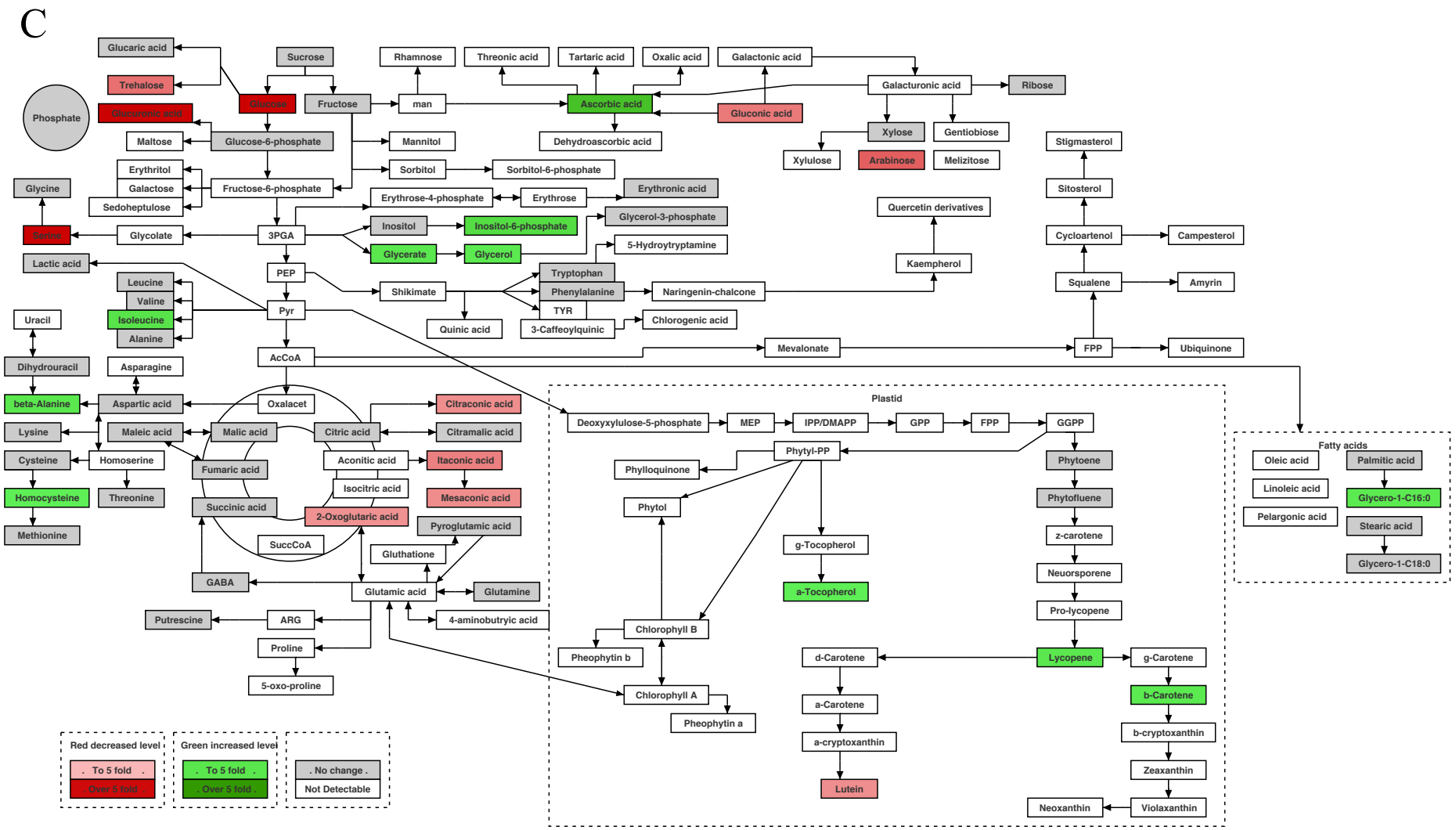
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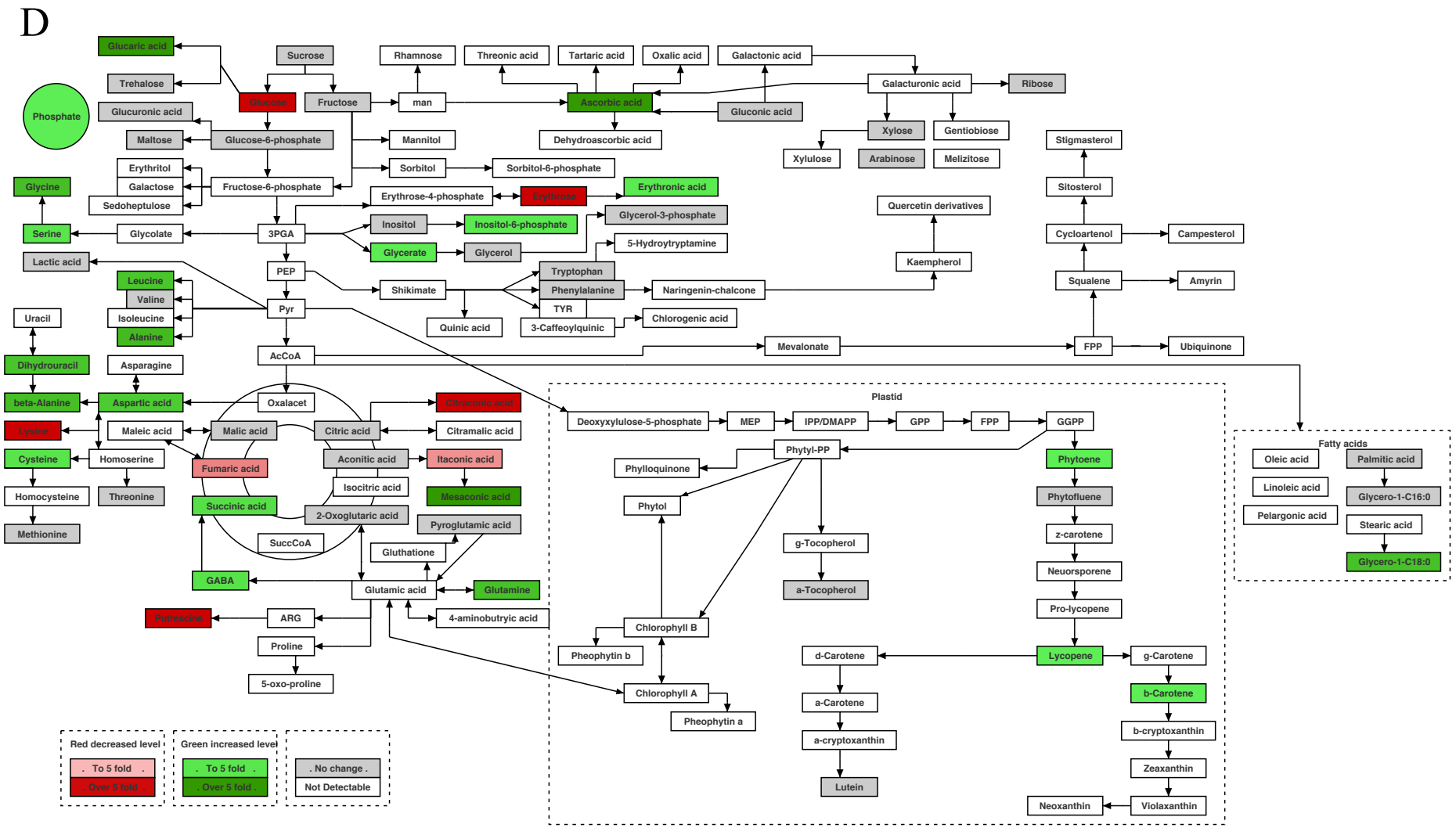
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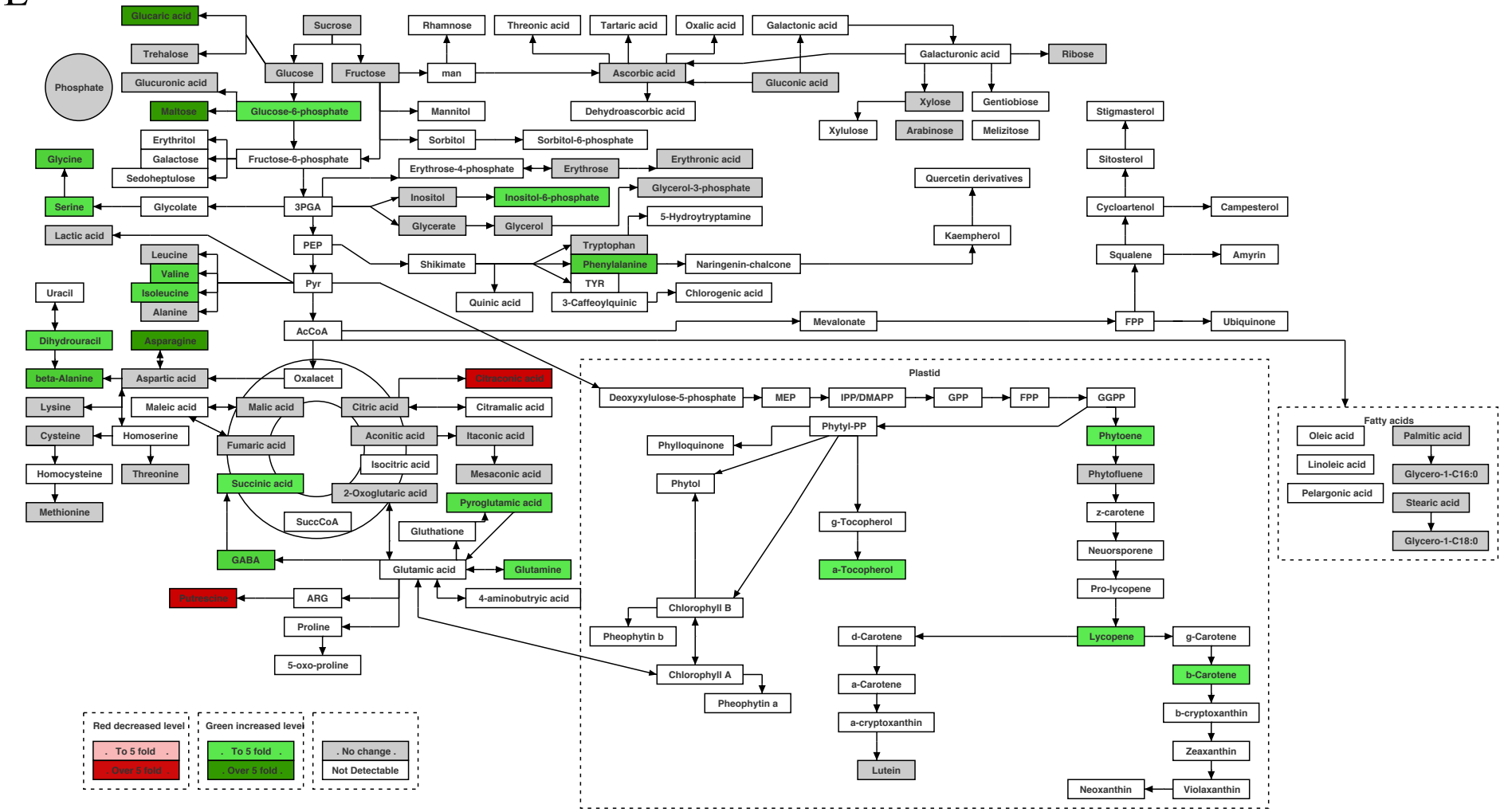



Figure 3-8 Pathway displays of metabolite changes in fruits of Q1968 to M82 control in different stages of fruit development and ripening

Figure 3-8 Pathway displays of metabolite changes in tomato fruits of the Q1968 compared to the M82 control at different stages of fruit development and ripening.

Pathway display of metabolite changes are illustrated in (A) Mature green stage, (B) 3 days post breaker, (C) 7 days post breaker, (D) 14 days post breaker, (E) Storage stage (7 days post breaker fruits stored at 4°C for 7 more days). The figures displayed metabolite changes quantitatively over schematic representations in biochemical pathway using BioSynLab software (www.biosynlab.com). The fold changes of compound levels are presented as ratio of Q1968 level to the M82 comparator. Light red indicates up to 5-fold decrease and dark red more than 5-fold decrease whereas light green illustrates up to 5-fold increase and dark green more than 5-fold increase. Grey shows no significant changes. White indicates that the metabolite was undetectable either in the samples or in the analytical platforms used. 3PGA, glyceraldehyde-3-phosphate; Ac-CoA, acetyl-coenzyme A; ARG, arginine; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl-pyrophosphate; GPP, geranyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; SuccCoA, succinyl-coenzyme A; TYR, tyrosine.

### 3.2.3.4 RNAseq analysis for Q1968 through stages of ripening

#### 3.2.3.4.1 The overview about the differentially expressed genes

In order to evaluate changes in the transcriptome, RNA-seq analysis was performed on the Q1968 line and compared to the M82 wild type control line as described in section 2.3.3.4. It was evident from the RNA-seq data that many of the transcripts displayed statistically significant differences between the two lines.

indicates the overall number of genes down regulated or up regulated during the different stages of fruit development and ripening (MG, 3dpb, 7dp b and considering all of data in all stages as one set of data together: all M82 against all Q1968).

Overall the number of up and down regulated transcripts found in the comparison between all the M82 data compared to all Q1968 data is nearly the same (around 3300 changes in transcripts; see Table 3-8). It was apparent that the largest changes in transcripts occurred at the MG stage representing around 2700 differentially expressed transcripts. With 2697 up regulated and 2856 down regulated transcripts the number of altered transcripts were similar. At the ripe stage the number of down regulated transcripts are almost doubled to those showing up regulation (see Table 3-8).

Table 3-8 Summary of differentially expressed transcripts determined at different stages of ripening and overall changes of Q1968 to M82.

"MG.Q1968.vs.M82", "BR3. Q1968.vs.M82" and "BR7.Q1968.vs.M82" represent Q1968 number of changed transcripts in comparison to M82 at mature green, 3 days post breaker and 7 days post breaker stages respectively. All. Q1968.vs.M82 indicate comparison of the total data of Q1968 at MG, 3dpb and ripe stage considered as one set of data and compared with all of M82 data. # Up and # Down means the number of genes upregulated or downregulated respectively.

| Comparison | # Up | # Down |
|---|---|---|
| MG.Q1968.vs.M82 | 2697 | 2856 |
| BR3. Q1968.vs.M82 | 693 | 603 |
| BR7.Q1968.vs.M82 | 621 | 1147 |
| All. Q1968.vs.M82 | 3306 | 3365 |

The Venn diagram compared the number of transcripts up and down regulated at different stages of ripening with common transcripts among them.

The 61 transcripts were up regulated in all stages of ripening while 98 were down regulated (Figure 3-8). These genes are substantial genes which affects all stages of ripening; on the contrary the specific genes for each stages of ripening are effective in that stages of ripening for example genes responsible for specific metabolites that produce in each stages of ripening. The overlapping segments of each two stage indicate the number of common transcripts (up and down regulated) between two stages.

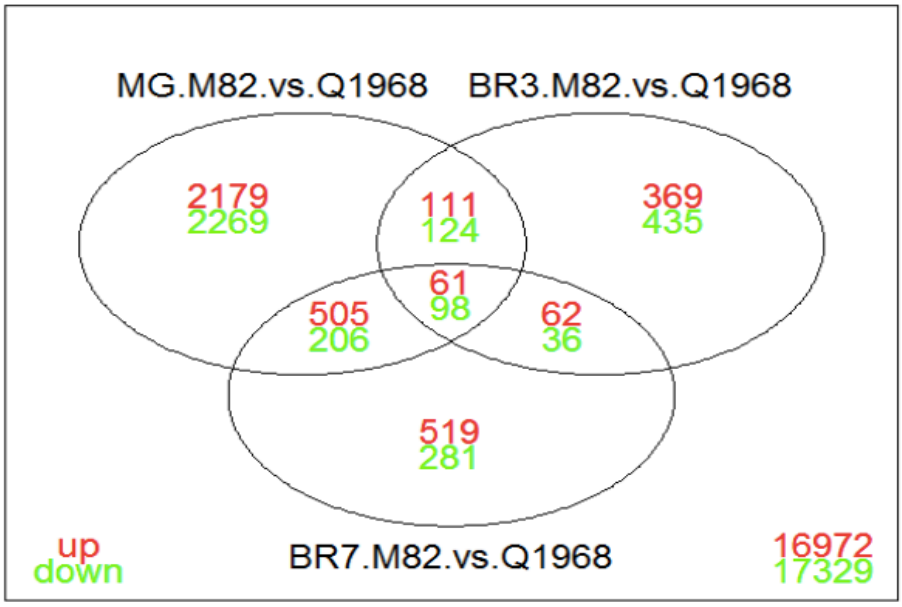



Figure 3-8 Venn diagram comparing the number of differential expressed genes between M82 to Q1968 in three stages of ripening.

Red color indicates the number of increased genes and green color shows the number of decreased genes. In the diagrams the number of common genes are presented in the overlapping segment. MG, BR3 and BR7 represent mature green, 3 dpb and 7 dpb stages of ripening respectively.

#### 3.2.3.4.2 The most differently expressed genes in the introgressed region of *S.pennellii* (Sub IL2-3/2-4) in Q1968 line

The sub IL of chromosome 2-3 and 2-4 from *S.pennellii* that exists in Q1968 theoretically is responsible for all of the phenotypic and the metabolic changes observed in Q1968 (see sections 3.2.3.1, 3.2.3.2 and 3.2.3.3). This could be in result of epistatic interaction of genes or parallel effects of some different genes. This region contained 64 genes with different putative functions. Some of these genes encode enzymes such as a putative transferase, helicase, GTPase, CTP synthase, endonuclease, pectinesterase and etc. Considerable number of genes encode protein binding proteins, DNA binding and ion binding proteins also observed. Among these 64 genes 4 genes with transcriptional or transcriptional related functions were detected (Solyc02g082010.1, Solyc02g081670.1, Solyc02g081780.1 and Solyc02g082040.2) (see section 3.3.2). In the RNA-seq data for 55 genes out of these 64 genes some data detected which 33 of them are expressed significantly different (based on FDR value) considering all stages of ripening. The most differently expressed genes of introgressed region considering all stages of ripening are shown in

Table 3-9. The detailed list of differently expressed genes of introgressed region in MG, 3dpb and ripe stages individually are reported in appendices 5, 6 and 7 in Table A 9-1, Table A 9-2 and Table A 9-3. Moreover, the top 20 differently expressed genes from overall transcriptome in MG, 3dpb and ripe stages of ripening separately and considering all stages together as one set were also reported in appendices 9 to 11; Table A 9-4, Table A 9-5, Table A 9-6 and Table A 9-7 for more detailed information. This is important to consider some of these genes are responsible for the reported changes in the Q1968 line. For conclusion and discussion see section 3.3.

Table 3-9 Top 20 differentially expressed genes of *S.Pennellii* introgressed region between M82 and Q1968 lines (comparison of all stages of ripening).

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The overall log2 fold change (log2FC) between M82 and Q1968 shown as All.M82.vs.All.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction presented as All.M82.vs.All.Q1968.FDR. Genes were considered statistically significantly differentially expressed if the FDR was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage. In this analysis all of the data for three stages of MG, 3dpb and 7dpb of M82 considered against the same data of Q1968.

| SOL ID | Description | GO term | All.M82.vs.All.Q1968.log2FC | All.M82.vs.All.Q1968.FDR |
|---|---|---|---|---|
| Solyc02g082100.2 | Neuralized (AHRD V1 *--- B4M452_DROVI); contains Interpro domain(s) IPR001841 Zinc finger, RING-type | Zinc ion binding | 4.48 | 8.96E-49 |
| Solyc02g081890.2 | Xylosyltransferase 1 (AHRD V1 **-- B6UEF2_MAIZE); contains Interpro domain(s) IPR003406 Glycosyl transferase, family 14 | Transferase activity, transferring glycosyl groups | -4.45 | 3.86E-04 |
| Solyc02g082190.2 | CTP synthase (AHRD V1 **** B6U7R1_MAIZE); contains Interpro domain(s) IPR004468 CTP synthase | CTP synthase activity; identical protein binding | -2.86 | 4.52E-56 |
| Solyc02g082120.2 | DNA-3-methyladenine glycosylase I (AHRD V1 ***- B6TPB7_MAIZE); contains Interpro domain(s) IPR005019 Methyladenine glycosylase | Base-excision repair; DNA repair | -2.81 | 3.57E-16 |
| Solyc02g081830.2 | HAD-superfamily hydrolase (AHRD V1 ***- D5A3P4_SPIPL); contains Interpro domain(s) IPR011949 HAD-superfamily hydrolase, subfamily IA, REG-2-Like | Protein binding | -2.29 | 4.76E-24 |
| Solyc02g081930.2 | Unknown Protein (AHRD V1) | . | -2.29 | 1.99E-09 |
| Solyc02g082180.2 | DNA replication licensing factor (AHRD V1 ***- Q2TWS7_ASPOR); contains Interpro domain(s) IPR008049 MCM protein 6 | Identical protein binding | -2.14 | 1.00E-24 |
| Solyc02g081850.2 | Amino acid transporter (AHRD V1 **** C2SWS0_BACCE); contains Interpro domain(s) IPR015606 Cationic amino acid transporter | High affinity arginine transmembrane transporter activity; cationic amino acid | -2.02 | 2.31E-17 |

| | | | | |
|---|---|---|---|---|
| | | transmembrane transporter activity | | |
| Solyc02g081860.1 | Pentatricopeptide repeat-containing protein (AHRD V1 ***- D7MRA3_ARALY); contains Interpro domain(s) IPR002885 Pentatricopeptide repeat | - | -1.96 | 4.85E-13 |
| Solyc02g081680.2 | Nucleolar complex protein 2 homolog (AHRD V1 ***- C0H9R3_SALSA); contains Interpro domain(s) IPR005343 Uncharacterised protein family UPF0120 | Identical protein binding | -1.88 | 4.38E-39 |
| Solyc02g082140.2 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR008166 Protein of unknown function DUF23 | - | 1.74 | 5.43E-26 |
| Solyc02g081970.2 | Vacuolar sorting receptor 7 (AHRD V1 **** B6UEQ5_MAIZE); contains Interpro domain(s) IPR003137 Protease-associated PA | Protein binding; mino-terminal vacuolar sorting propeptide binding | 1.57 | 6.09E-22 |
| Solyc02g082210.2 | Genomic DNA chromosome 3 P1 clone MOJ10 (AHRD V1 ***- Q9LSE0_ARATH) | - | 1.34 | 3.47E-12 |
| Solyc02g082060.1 | PPPDE peptidase domain-containing protein 1 (AHRD V1 *--- PPDE1_XENLA); contains Interpro domain(s) IPR008580 Protein of unknown function DUF862, eukaryotic | - | -1.32 | 5.76E-08 |
| Solyc02g081730.2 | Sterol-4-alpha-carboxylate 3-dehydrogenase decarboxylating (AHRD V1 **** B6TKP9_MAIZE); contains Interpro domain(s) IPR002225 3-beta hydroxysteroid dehydrogenase/isomerase | Sterol-4-alpha-carboxylate 3-dehydrogenase (decarboxylating) activity | 1.31 | 7.57E-06 |
| Solyc02g082130.1 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR007019 Surfeit locus 6 | - | -1.24 | 2.15E-16 |
| Solyc02g081880.2 | Molybdenum cofactor sulfurase protein-Like (AHRD V1 **-- Q653D7_ORYSJ); contains Interpro domain(s) IPR005303 MOSC, N-terminal beta barrel | Phospholipid-translocating ATPase activity | 1.23 | 3.45E-16 |
| Solyc02g081940.2 | RNA binding protein (AHRD V1 **** D8LH81_ECTSI); contains Interpro domain(s) IPR012677 Nucleotide-binding, alpha-beta plait | Helicase activity; mRNA binding | -1.22 | 1.37E-12 |
| Solyc02g082110.2 | Pre-mRNA-splicing factor cwc24 (AHRD V1 **-- B2WLF6_PYRTR); contains Interpro domain(s) IPR000571 Zinc finger, CCCH-type | Zinc ion binding | -1.15 | 1.61E-11 |
| Solyc02g082160.1 | Pentatricopeptide repeat-containing protein (AHRD V1 ***- D7KMD0_ARALY); contains Interpro domain(s) IPR002885 Pentatricopeptide repeat | Endonuclease activity | -1.1 | 4.46E-04 |

#### 3.2.3.4.3 Selected genes showing the most significant differential expression (up and down regulated) within the Q1968 transcriptome compared to M82

The introgressed region had a significant effect on the overall transcriptome. These genes have different biological properties and functions (The scientific explanation about the role of each transcript noted in footnote for general information about them). The most differentially expressed genes in the overall transcriptome of M82 line compared to Q1968 line (Table 3-10 & Table 3-11) can be divided in three categories:

1) Group one, contains many transcripts encoding components of chlorophyll biosynthesis, some anabolic pathways, carbon fixation and DNA replication. For instance the altered transcripts with the following GO terms: chlorophyll a biosynthesis II, carbon fixation, acetyl CoA metabolic process, thiamine biosynthetic process[1], response to sucrose, sucrose metabolic process, glycogen biosynthetic process and glycerol-3-phosphate catabolic process[2] represent alteration of carbon fixation and anabolic biosynthesis pathways. The two changed ontology terms of DNA replication and reductive pentose phosphate cycle[3] suggest the changes in cell division as well (Table 3-10 & Table 3-11). Changes in gibberellin biosynthesis III and superpathway of gibberellin biosynthesis could affect cell division and elongation[4] (Table 3-10 & Table 3-11).

2) The second group represent transcripts correlating with cell wall effects and ripening. Pectin catabolic process and cellulose biosynthesis changes imply their effects via cell wall alterations, and also pathways affecting ethylene production like: Cysteine biosynthetic process from serin[5], SAM (S-adenosylmethionine) biosynthetic process and ethylene-activated signalling pathway. Both of these gene categories affect ripening (Table 3-10 & Table 3-11).

---

[1] Thiamine is an important coenzyme for 2 important enzymes in TCA cycle.

[2] Glycerol-3-phosphate catabolic process transferring cytosolic NADH energy to mitochondrial forms of FADH.

[3] Pentose phosphate cycle is active in anabolic process of ribose-5-phosphate production for nucleic acid biosynthesis and also NADPH production.

[4] It is demonstrated that treatment with GA3 elevated the expression of genes correlating with DNA replication effecting histones h1 and h2b, and also cell elongation via expansin and α-tubulin (van den Heuvel et al., 2001)

[5] Cysteine is provider of sulphur (S) for methionine production, which is a precursor for ethylene.

3) Third group contains a variety of transcripts predominantly from biochemical pathways;

$3_i$) Some pathway components responsible for production of secondary metabolites. This includes phenylpropanoid biosynthesis[1], L-phenylalanine catabolic process[2] and monolignol glucosides biosynthesis[3] (Table 3-10 & Table 3-11).

$3_{ii}$) Another set of transcripts were linked to plant defence processes such as oxylipins[4] and response to chitin[5]. Trehalose[6] degradation II gene is another example of transcripts linked to plant defence processes.

$3_{iii}$) Third sub category consists of transcripts related to lipid biosynthesis and membrane lipids such as glycolipid desaturation[7], lipid desaturation synthesis, saponins I & II biosynthesise[8], oleate biosynthesis I, petroselinate biosynthesis with role in unsaturated fatty acid biosynthesis, *cis*-vaccenate biosynthesis affects unsaturated fatty acid biosynthesis and phospholipid desaturation.

$3_{iv}$) Lastly, the fourth sub groups affecting seedling growth and germination contained inositol biosynthesis[9], lipid-independent phytate[10] biosynthesis (Table 3-11).

[1] Phenylpropanoid produce important secondary metabolites of phenylpropanoid esters, flavonoids, anthocyanin and lignin (Ali and McNear, 2014).
[2] L-phenylalanine catabolic process provide precursor of phenylpropanoids.
[3] Monolignol glucosides is used in biosynthesis of lignin.
[4] Oxylipins act in wound healing and have anti stress effects (Howe and Schilmiller, 2002).
[5] Chitin, a polymer of N-acetyl-D-glucosamine, is a section of the fungal cell wall, which is not detected in plants. Plant cells with chitin degrading enzymes are able for digesting fungal cell walls (Wan et al., 2008).
[6] Trehalose, which is a potential signal metabolite in plants, also interacts with pathogenic or symbiotic herbivorous insects and microorganisms. Trehalose is also implicated in responses to cold and salinity as well (Lunn et al., 2014).
[7] Glycolipids desaturation pathway involve in biosynthesis of 18:3-16:3-MGDG and 18:3-18:3-DGDG which are the two most frequent glycosylglycerolipid found in chloroplast membranes (Mekhedov et al., 2000).
[8] Saponins I & II detected in glycoside lipids.
[9] Inositol tacke parts for myo-inositol biosynthesis.
[10] Phytate is the storage form of myo-inositol, mineral nutrients and phosphate for usage in seed germination and seedling growth (Raboy, 2001).

Table 3-10 Grouping of top significantly enriched GO Terms based on their functions between M82 and Q1968 lines (comparison of all stages of ripening) in the whole transcriptome.

GO term indicate the Gene Ontology. Each GO terms include different Genes which represent in Table 3-11. Grouping of genes were performed manually on the top 20 differently expressed genes on the whole transcriptome based on their GO terms.

| | Group name | Categories | Example of GO terms |
|---|---|---|---|
| Group 1 | Anabolic pathway and cell division | Chlorophyll biosynthesis and carbon fixation | Chlorophyll a biosynthesis II, Carbon fixation,<br>Acetyl CoA metabolic process, Thiamine biosynthetic process |
| | | Anabolic pathways | Response to sucrose, Sucrose metabolic process, Glycogen biosynthetic process, Glycerol-3-phosphate catabolic process |
| | | DNA replication | DNA replication,<br>Reductive pentose phosphate cycle |
| | | Cell division and elongation hormones | Gibberellin biosynthesis III,<br>Superpathway of gibberellin biosynthesis |
| Group 2 | Cell wall components and ripening | Cell wall effects | Pectin catabolic process,<br>Cellulose biosynthesis changes |
| | | Ripening effects | Cysteine biosynthetic process from serine,<br>SAM (S-adenosylmethionine) biosynthetic process,<br>Ethylene-activated signalling pathway |
| Group3 | Different biochemical pathways | Secondary metabolites production | Phenylpropanoid biosynthesis, L-Phenylalanine catabolic process,<br>Monolignol glucosides biosynthesis. |
| | | Plant defence processes | Oxylipins,<br>Response to chitin<br>Trehalose degradation II |
| | | Lipid biosynthesis and membrane lipids | Glycolipid desaturation, Oleate biosynthesis I, Petroselinate biosynthesis, *Cis*-vaccenate biosynthesis, Phospholipid desaturation. |
| | | Seedling growth and germination | Inositol biosynthesis<br>Lipid-independent phytate biosynthesis |

Table 3-11 Selected top significantly enriched pathways (A) and selected top significantly enriched GO Terms (B) from the comparison of M82 and Q1968 (comparison of all stages of ripening) in the whole transcriptome.

The ontology term (GO) (table A) and biochemical pathway (table B) represent the biochemical process/pathway which some genes involving in this process were detected in the whole transcriptome. Differently expressed gene represents the sol ID of differently expressed genes. NO Genes indicate the number of total detected genes whether their expression changed or not. No of changed gens shows the number of changed genes and % of changed genes represent the number of changed genes as percentage of number of total detected genes. Gene set enrichment analysis was performed using ROAST. Gene sets were considered enriched if the FDR (false discovery rate) from testing the mixed hypothesis, which does not depend on the directionality of the differential expression, was <0.05 and the gene set contained at least one differentially expressed gene. The number of genes that were statistically differentially expressed from the overall detected genes for different GO terms and Biochemical pathways are shown in table A and B respectively.

A

| GO Term | Differentially Expressed Genes | No Genes | No of changed genes | % of changed genes | FDR |
|---|---|---|---|---|---|
| DNA replication initiation | Solyc01g079500.2, Solyc01g110130.2, Solyc02g082180.2, Solyc02g070780.2, Solyc07g005020.2, Solyc11g040120.1 | 7 | 6 | 86% | 1.69E-05 |
| Inositol biosynthetic process | Solyc04g014800.2, Solyc04g050820.1, Solyc04g054740.2, Solyc05g051850.2 | 5 | 4 | 80% | 5.87E-05 |
| Pectin catabolic process | Solyc02g093580.2, Solyc03g123630.2, Solyc07g064190.1, Solyc07g064180.2 | 5 | 4 | 80% | 5.87E-05 |
| Thiamine biosynthetic process | Solyc01g103950.2, Solyc05g053480.2, Solyc06g006080.2, Solyc07g064160.2 | 5 | 4 | 80% | 5.87E-05 |
| Coenzyme A metabolic process | Solyc02g038740.2, Solyc03g032020.2, Solyc03g032010.2 | 4 | 3 | 75% | 5.87E-05 |
| GDP-mannose biosynthetic process | Solyc02g063220.2, Solyc06g060100.2, Solyc08g008670.2 | 4 | 3 | 75% | 5.87E-05 |

| | | | | | |
|---|---|---|---|---|---|
| Glycerol-3-phosphate catabolic process | Solyc01g088150.2, Solyc02g067930.2, Solyc04g016330.2 | 4 | 3 | 75% | 5.87E-05 |
| S-adenosylmethionine biosynthetic process | Solyc01g101060.2, Solyc09g008280.1, Solyc10g083970.1 | 4 | 3 | 75% | 5.87E-05 |
| Reductive pentose-phosphate cycle | Solyc02g085950.2, Solyc02g063150.2, Solyc03g034220.2 | 4 | 3 | 75% | 1.69E-05 |
| Glutamine biosynthetic process | Solyc04g014510.2, Solyc10g083830.1, Solyc11g011380.1 | 4 | 3 | 75% | 5.87E-05 |
| Sucrose metabolic process | Solyc02g081300.2, Solyc03g098290.2, Solyc03g083910.2, Solyc07g042550.2, Solyc07g007790.2, Solyc09g098590.2, Solyc11g045110.1, Solyc12g009300.1, Solyc12g040700.1 | 12 | 9 | 75% | 5.87E-05 |
| Cysteine biosynthetic process from serine | Solyc01g094790.2, Solyc01g097920.2, Solyc01g097950.2, Solyc02g082850.2, Solyc07g065470.2, Solyc07g065340.1, Solyc09g082060.2, Solyc10g006040.1 | 11 | 8 | 73% | 5.87E-05 |
| Carbon fixation | Solyc02g085950.2, Solyc02g063150.2, Solyc03g034220.2, Solyc04g006970.2, Solyc07g062530.2, Solyc10g007290.2, Solyc12g014250.1 | 10 | 7 | 70% | 5.87E-05 |
| Ethylene-activated signalling pathway | Solyc03g093610.1, Solyc03g097920.1, Solyc05g055070.2, Solyc06g053710.2, Solyc07g056580.2, Solyc09g089610.2, Solyc11g073050.1 | 10 | 7 | 70% | 5.87E-05 |
| Response to chitin | Solyc01g098410.2, Solyc02g093050.2, Solyc03g116890.2, Solyc04g077980.1, | 10 | 7 | 70% | 5.87E-05 |

| | | | | | |
|---|---|---|---|---|---|
| | Solyc06g005170.2, Solyc07g040690.2, Solyc08g006320.2 | | | | |
| Response to sucrose | Solyc01g107730.2, Solyc05g007180.2, Solyc06g059840.2, Solyc06g007180.2, Solyc10g083610.1 | 7 | 5 | 71% | 5.87E-05 |
| Oxylipin biosynthetic process | Solyc01g099190.2, Solyc03g122340.2, Solyc07g007870.2, Solyc08g014000.2, Solyc10g086220.1 | 7 | 5 | 71% | 5.87E-05 |
| Glycogen biosynthetic process | Solyc01g109790.2, Solyc01g079790.2, Solyc07g056140.2, Solyc09g009190.2 | 6 | 4 | 67% | 5.87E-05 |
| Glycerol-3-phosphate metabolic process | Solyc01g088150.2, Solyc02g067930.2, Solyc04g016330.2, Solyc11g008380.1 | 6 | 4 | 67% | 5.87E-05 |
| L-phenylalanine catabolic process | Solyc03g042560.1, Solyc09g007910.2, Solyc09g007890.1, Solyc09g007920.2, Solyc09g007900.2, Solyc10g086180.1 | 9 | 6 | 67% | 5.87E-05 |
| Calcium ion transport | Solyc01g096190.2, Solyc03g123790.2, Solyc09g005260.2, Solyc12g055750.1 | 6 | 4 | 67% | 5.87E-05 |
| Monolignol glucosides biosynthesis | Solyc02g085660.1, Solyc05g053120.1, Solyc05g053400.1, Solyc05g012670.1, Solyc09g059170.1 | 7 | 5 | 71% | 1.64E-05 |
| Trehalose degradation II (trehalase) | Solyc02g091830.2, Solyc03g121070.2, Solyc04g081400.2, Solyc11g065220.1, Solyc12g008510.1 | 7 | 5 | 71% | 5.52E-05 |
| Saponin biosynthesis I | Solyc01g105350.1, Solyc02g091350.2, Solyc03g078490.2, Solyc03g078730.1, Solyc03g083650.2, Solyc03g078770.2, Solyc04g074350.2, Solyc05g055840.2, Solyc07g006800.1, Solyc12g006510.1, Solyc12g006520.1 | 16 | 11 | 69% | 5.52E-05 |

| | | | | | |
|---|---|---|---|---|---|
| Saponin biosynthesis II | Solyc01g105350.1, Solyc02g091350.2, Solyc03g078490.2, Solyc03g078730.1, Solyc03g083650.2, Solyc03g078770.2, Solyc04g074350.2, Solyc05g055840.2, Solyc07g006800.1 | 13 | 9 | 69% | 5.52E-05 |
| Phenylpropanoid biosynthesis, initial reactions | Solyc03g078270.1, Solyc03g036470.1, Solyc03g042560.1, Solyc05g047530.2, Solyc09g007910.2, Solyc09g007890.1, Solyc09g007920.2, Solyc09g007900.2, Solyc10g086180.1 | 13 | 9 | 69% | 5.52E-05 |
| Coniferin metabolism | Solyc02g085660.1, Solyc02g080300.2, Solyc02g080310.2, Solyc02g080290.2, Solyc05g053120.1, Solyc05g053400.1, Solyc05g012670.1, Solyc09g059170.1 | 12 | 8 | 67% | 5.52E-05 |
| Molybdenum cofactor (sulfide) biosynthesis | Solyc01g044370.1, Solyc02g081880.2, Solyc03g031420.1, Solyc07g066480.2 | 6 | 4 | 67% | 5.52E-05 |
| Chlorophyll a biosynthesis II | Solyc01g088310.2, Solyc07g064660.1, Solyc09g008920.2, Solyc11g012850.1 | 6 | 4 | 67% | 5.52E-05 |
| Lipid-independent phytate biosynthesis | Solyc03g097410.1, Solyc04g077120.2, Solyc04g050820.1, Solyc04g054740.2, Solyc05g051850.2, Solyc06g072740.1, Solyc08g015630.2, Solyc08g076690.1 | 12 | 8 | 67% | 5.52E-05 |
| Cellulose biosynthetic process | Solyc01g087210.2, Solyc02g072240.2, Solyc03g097050.2, Solyc03g070390.2, Solyc03g005450.2, Solyc04g071650.2, | 23 | 15 | 65% | 5.87E-05 |

| | | | | | |
|---|---|---|---|---|---|
| | Solyc05g012070.2, Solyc07g005840.2, Solyc07g065660.2, Solyc08g082640.2, Solyc08g076320.2, Solyc08g061100.2, Solyc11g005560.1, Solyc12g014430.1, Solyc12g056580.1 | | | | |

B

| **Pathway Name** | **Differentially Expressed Genes** | **No of Genes** | **No of changed genes** | **% of changed genes** | **FDR** |
|---|---|---|---|---|---|
| Glycerol-3-phosphate shuttle | Solyc01g088150.2, Solyc02g067930.2, Solyc04g016330.2, Solyc11g008380.1, Solyc11g008370.1 | 5 | 5 | 100% | 5.52E-05 |
| Gibberellin biosynthesis III (early C-13 hydroxylation) | Solyc02g062470.1, Solyc02g062490.2, Solyc06g066820.2, Solyc07g061730.2, Solyc11g072310.1 | 6 | 5 | 83% | 5.52E-05 |
| Superpathway of gibberellin biosynthesis | Solyc02g062470.1, Solyc02g062490.2, Solyc06g066820.2, Solyc07g061730.2, Solyc11g072310.1 | 6 | 5 | 83% | 5.52E-05 |
| Glycolipid desaturation | Solyc01g006430.2, Solyc04g040130.1, Solyc06g007130.2, Solyc06g007140.2, Solyc12g044950.1 | 6 | 5 | 83% | 5.52E-05 |
| Phospholipid desaturation | Solyc01g006430.2, Solyc04g040130.1, Solyc06g007130.2, Solyc06g007140.2, Solyc12g044950.1 | 6 | 5 | 83% | 5.52E-05 |
| Petroselinate biosynthesis | Solyc05g014560.2, Solyc06g083380.2, Solyc10g080890.1, Solyc12g006930.1 | 5 | 4 | 80% | 5.52E-05 |

| | | | | | |
|---|---|---|---|---|---|
| Cis-vaccenate biosynthesis | Solyc05g014560.2, Solyc06g083380.2, Solyc10g080890.1, Solyc12g006930.1 | 5 | 4 | 80% | 5.52E-05 |
| S-adenosylmethionine biosynthesis | Solyc01g094190.2, Solyc01g101060.2, Solyc09g008280.1, Solyc10g083970.1 | 5 | 4 | 80% | 5.52E-05 |
| Acetyl-CoA biosynthesis (from citrate) | Solyc01g101040.2, Solyc01g059880.2, Solyc05g005160.2 | 4 | 3 | 75% | 5.52E-05 |
| Phytyl diphosphate biosynthesis | Solyc01g088310.2, Solyc07g064660.1, Solyc09g008920.2 | 4 | 3 | 75% | 5.52E-05 |
| Ammonium transport | Solyc03g045070.1, Solyc07g025490.1, Solyc10g076480.1 | 4 | 3 | 75% | 5.52E-05 |
| Melibiose degradation | Solyc03g019790.2, Solyc05g013720.2, Solyc06g050130.2 | 4 | 3 | 75% | 5.52E-05 |
| Glutamine biosynthesis | Solyc04g014510.2, Solyc10g083830.1, Solyc11g011380.1 | 4 | 3 | 75% | 5.52E-05 |
| Cysteine biosynthesis I | Solyc01g094790.2, Solyc01g097920.2, Solyc01g097950.2, Solyc02g082850.2, Solyc07g065470.2, Solyc07g065340.1, Solyc09g082060.2, Solyc10g006040.1 | 11 | 8 | 73% | 5.52E-05 |
| Oleate biosynthesis I (plants) | Solyc06g059710.2, Solyc06g053480.2, Solyc06g083380.2, Solyc06g059720.2, Solyc12g006930.1 | 7 | 5 | 71% | 5.52E-05 |
| Ammonia assimilation cycle II | Solyc03g083440.2, Solyc04g014510.2, Solyc08g044270.2, Solyc10g083830.1, Solyc11g011380.1 | 7 | 5 | 71% | 5.52E-05 |
| Trehalose degradation II (trehalase) | Solyc02g091830.2, Solyc03g121070.2, Solyc04g081400.2, Solyc11g065220.1, Solyc12g008510.1 | 7 | 5 | 71% | 5.52E-05 |
| Monolingual glucosides biosynthesis | Solyc02g085660.1, Solyc05g053120.1, Solyc05g053400.1, Solyc05g012670.1, Solyc09g059170.1 | 7 | 5 | 71% | 1.69E-05 |
| Phenylpropanoid biosynthesis, initial reactions | Solyc03g078270.1, Solyc03g036470.1, Solyc03g042560.1, Solyc05g047530.2, Solyc09g007910.2, Solyc09g007890.1, | 13 | 9 | 69% | 5.52E-05 |

| | | | | | |
|---|---|---|---|---|---|
| | Solyc09g007920.2, Solyc09g007900.2, Solyc10g086180.1 | | | | |
| Saponin biosynthesis I | Solyc01g105350.1, Solyc02g091350.2, Solyc03g078490.2, Solyc03g078730.1, Solyc03g083650.2, Solyc03g078770.2, Solyc04g074350.2, Solyc05g055840.2, Solyc07g006800.1, Solyc12g006510.1, Solyc12g006520.1 | 16 | 11 | 69% | 5.52E-05 |
| Saponin biosynthesis II | Solyc01g105350.1, Solyc02g091350.2, Solyc03g078490.2, Solyc03g078730.1, Solyc03g083650.2, Solyc03g078770.2, Solyc04g074350.2, Solyc05g055840.2, Solyc07g006800.1 | 13 | 9 | 69% | 5.52E-05 |

The expressions of some important transcripts that are known to affect ripening were altered. These include master transcription factors such as *RIN, NOR, CNR* and *NR*. Other ripening related transcripts from genes encoding components of the ethylene biosynthetic pathway and cell wall modifying enzymes such as polygalacturonase were extracted from RNA-seq data and changes from the control levels are illustrated in (Figure 3-9).

*CNR, NR* and *NOR* TFs show considerable increases in Q1968 compared to M82 at the MG stage. As Table 3-11 shows ethylene-activated signalling pathway representing 70% changes in the pattern of gene expression between the two lines of M82 and Q1968. *ACO1* and *ACS2* are important genes in biosynthesis of ethylene, which highly up-regulated at MG stage in Q1968 compared to M82 line. At ripe stage *ACS2* is also upregulated, but on the contrary *ACO1* is down regulated in Q1968 to M82. It is interesting that most of changes, even the transcripts that suppose to upregulated at later stages, represent their alterations at MG stage (Figure 3-9 A). The Q1968 line is a candidate for a high lycopene sub-IL, the expression of carotenoid genes were illustrated from RNA-seq data in Figure 3-9 B. Most of the carotenoid genes (*DXS, PSY1, PSY2, CRTISO, LYCB* and *CRTR B1*) represent a considerable increase at MG stage, but this trend was not observed at the 3dpb and ripe stages (see Figure 3-9 B). Fruit specific lycopene cyclase was highly downregulated at ripe stage, which helps in accumulation more lycopene in Q1968 to M82 wild type. But surprisingly *PSY*2 (leaf specific) is highly downregulated in 3 dpb and ripe stages in Q1968 to M82, which does not correlate firstly with the fact that transcription of carotenoid genes in a specific tissue is strongly under regulation on that tissue and the alternative tissue genes, which does not usually express in that tissue, undergoing the desired alterations and are not under the same regulatory mechanism (Fray et al., 1995) and secondly with higher lycopene production in Q1968; therefore such a high silence of *PSY2* in Q1968 to M82 was not expected at 3dpb and ripe stages.

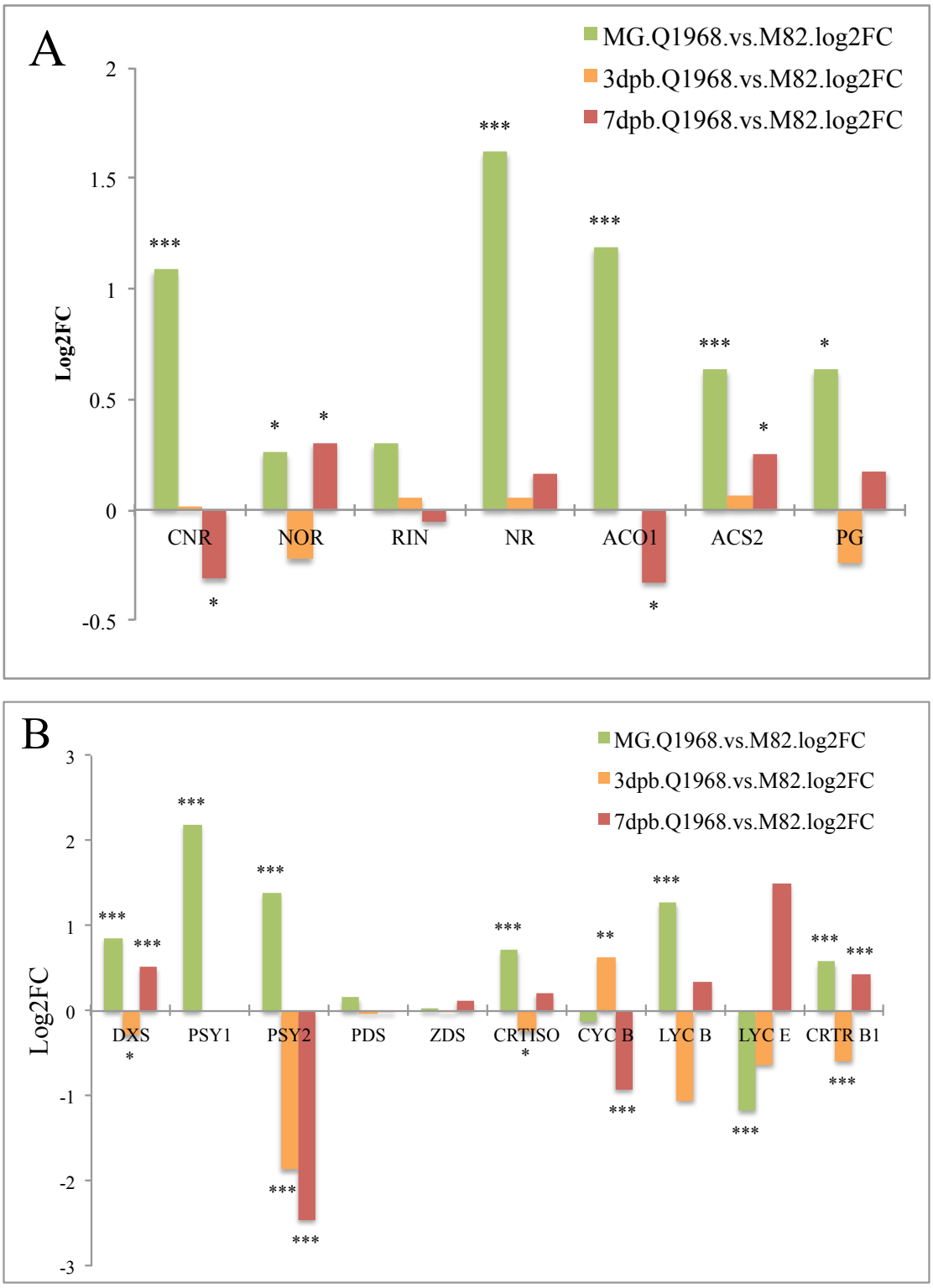



Figure 3-9 The expression levels of some selected genes affecting ripening (A) and carotenoid biosynthetic genes (B) from the comparison of M82 and Q1968 transcriptome extracted from RNA-seq data in different stages of ripening.

The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between Q1968 and M82 at MG stage shown

as “MG.Q1968.vs. M82.log2FC” and for the 3dpb and ripe stage shown as 3dpb.”Q1968.vs. M82.log2FC” and “7dpb.Q1968.vs. M82.log2FC” respectively. The false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at MG stage presented as MG.M82.vs.Q1968.FDR Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. *NR*, never-ripe; *RIN*, ripening-inhibitor; *NOR*, non-ripening; *CNR*, colourless non-ripening; *ACO1*, ACC oxidase; *ACS2*, ACC synthase; *PG*, polygalacturonase; *DXS*, 1-deoxy-D-xylulose-5- phosphate synthase; *PSY1*, phytoene synthase-1; *PSY2*, phytoene synthase-1; *PDS*, phytoene desaturase; *ZDS*, z-carotene desaturase; *CRTISO*, carotene isomerase; *CYC B*, b-lycopene cyclase; *LCY B*, b-lycopene cyclase; (11) *LCY E*, e-lycopene cyclase; *CRTR B1*, β-Carotene hydroxylase.

## 3.3 Discussion

### 3.3.1 A combination of genes in the introgressed region could be responsible for the overall changes in the transcriptome

Considering the large variation in the types of genes represented in the introgressed region of subIL2-3 & 2-4 (Table 3-9) and also the impressive impact on the expression pattern of other genes in the whole genome (Table 3-10 & Table 3-11), elucidating an underlying mechanism of action does not seem simple. The first plausible mechanisms could be in result of correlations among some functional/structural genes in the region; this means the effect of some of differently expressed gene in the introgressed region could result in the vast changes in the overall transcriptome. Because some relation detected among changed gene in the introgressed region and the other most changed genes in overall transcriptome. Some of altered transcripts in the region correlate with the first category of genes (Table 3-10) relating to carbon fixation, overall changes in anabolic pathway, DNA replication and cell division.

As the first example for first group of genes the 5 following genes in the region: DNA replication licensing factor (Solyc02g082180.2), structural constituent of ribosome which is 30S ribosomal protein S19 (Solyc02g082000.2), enzyme with helicase activity and mRNA binding (Solyc02g081940.2), DNA repair (Solyc02g082120.2) and endonuclease activity (Solyc02g081900.2) could trigger the later feedback on changing related transcripts in overall transcriptome such as DNA replication

initiation genes that present 86% different in their expression (Table 3-11) and reductive pentose phosphate cycle[1] with 75% changes (Table 3-11).

As the second example for first groups of genes, CTP synthase activity (Solyc02g082190.2) altered transcript in the region could activate the pathway effecting pyrimidine biosynthesis GOs. For example ammonium transport[2] with 75% changes (Table 3-11); glutamine biosynthesis[3] with75% change and finally reductive pentose phosphate cycle with 75% alteration in the transcripts (Table 3-10 & Table 3-11).

The third instance for altered transcripts from first category of genes could be the 4 following altered transcripts of plastid ribulose-1, 5 bisphosphate carboxylase/oxygenase large subunit N-methyltransferase-related protein (Solyc02g081920.2), ATPase activity (Solyc02g081980.2), GTPase activity (Solyc02g081660.2) and cytochrome P450[4] (Solyc02g082070.2) in the region which could be responsible or have some connections for overall changes in ontology terms such as gibberellin biosynthesis III (83% changes), superpathway of gibberellin biosynthesis (83% alteration), chlorophyll a biosynthesis II (67% changes) and carbon fixation (70% alteration).

As the forth example of first category of genes molybdenum cofactor sulfurase protein-Like (Solyc02g081880.2) in the introgressed region could have effects on 67% changes of genes expression under molybdenum cofactor (sulfide) biosynthesis ontology term.

The second group of altered genes, which affecting ripening, only had one representative in the sub-IL2-3 & 2-4; Solyc02g081990.2 with pectinesterase activity effecting cell wall degradation.

In the third group of genes, which one category was related to lipid biosynthesis and membrane lipids (Table 3-10) such as glycolipid desaturation and phospholipid desaturation (both GO changed 83%), might be affected by some differently expressed genes in the region. For instance, acyltransferase (Solyc02g081800.1),

[1] Pentose phosphate cycle is active in anabolic process of ribose-5-phosphate production for nucleic acid biosynthesis and also NADPH production.

[2] Ammonium transport pathway plays role for providing the amine group of glutamate.

[3] Glutamine biosynthesis process produces the precursor in pyrimidine biosynthesise.

[4] Conversions of geranylgeranyl diphosphate into bioactive GAs consist of three classes of enzymes which one of them is membrane-bound cytochrome P450 mono oxygenases (P450s) (Kobayashi et al., 1988).

xylosyltransferase1 which transferring glycosyl groups (Solyc02g081890.2), phospholipid-translocating ATPase activity (Solyc02g081880.2).

If in overall one gene in the region wanted to be considered as a main or important effector to overall transcriptome, Solyc02g082070.2 represent Cytochrome P450 with oxygen binding GO could be a good candidate gene for Q1968 phenotype. The expression of this gene was 1.12 Log2FC in Q1968 to M82 control at MG stage which means 2.18 fold increase in its expression; considering broad function of P450 in different enzymes, this gene also could be considered in more detail as a candidate gene. The role of P450 have shown in different enzymes for instance:

1- β-ring carotene hydroxylase (including the non-heme di-iron type HYDB and the cytochrome P450 type CYP93A) and ε-ring carotene hydroxylase (the cytochrome P450 type CYP93C) convert beta and alpha carotene to lutein redundantly (Figure 3-10 represent the role of CYP93A in carotenoid biosynthesis pathway) (Tian et al., 2004; Galpaz et al., 2006; Kim and DellaPenna, 2006).

2- ABA catabolism with its role in seed imbibition (Kushiro et al., 2004).

3- Genes with role in drought stress (Kushiro et al., 2004).

4- Brassinosteroid hormones with its role in protection against cold and drought stress and cell division and cell elongation (Ohnishi et al., 2006; Shimada et al., 2001; Clouse and Sasse, 1998),

5- oxylipins and their defensive effects (Howe et al., 2014; Howe and Schilmiller, 2002).

Some of the mentioned enzymes, hormones and metabolites that had the role of P450 in their function, represent considerable change in their expression pattern in Q1968 line to M82. For example, oxylipins with 71% changes, in differently of expressed genes, brassinosteroid mediated signalling pathway (67%), brassinosteroid biosynthesis II (50%), response to abscisic acid (55%) correlate with this possibility. It is plausible the altered genes with defensive and anti stress activity, explained in this group of genes and in the second sub category, have changed in result of cytochrome P450 (Solyc02g082070.2) alteration in the introgressed region or cytochrome P450 imply a broader effect. Therefore, cytochrome P450 (Solyc02g082070.2) gene can be counted as a strong candidate genes result in some Q1968 altered characteristics. This hypothesis needs more researches to be validated (see section 6.2).

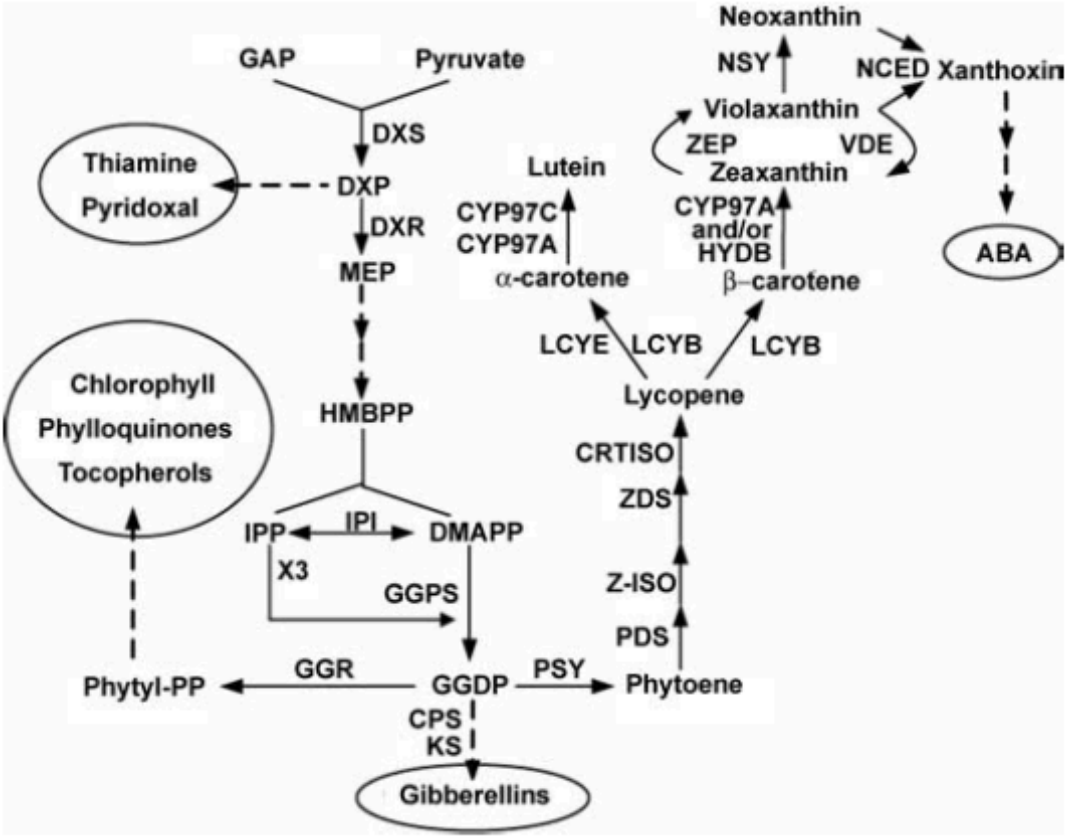



Figure 3-10 Carotenoid metabolic pathway in plants.

"Carotenoid biosynthesis begins with the synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via the 2-C-methyl- D-erythritol 4-phosphate (MEP) pathway using glyceraldehyde-3-phosphate (GAP) and pyruvate as initial substrates. IPP and DMAPP are used to form the central intermediate geranylgeranyl diphosphate (GGDP) for carotenoid and other isoprenoid biosynthesis via the general isoprenoid biosynthetic pathway. The synthesis of phytoene by condensation of two molecules of GGDP represents the first committed step in the carotenoid biosynthetic pathway. ABA, abscisic acid; CPS, ent-copalyl diphosphate synthase; CRTISO, carotene isomerase; CYP93A, carotene β-hydroxylase (cytochrome P450 type); CYP93C, carotene ε-hydroxylase (cytochrome P450 type); DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GGPS, geranylgeranyl diphosphate synthase; GGR, geranylgeranyl diphosphate reductase; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; HYDB, carotene β-hydroxylase (non- heme di-iron type); IPI, isopentenyl diphosphate isomerase; KS, ent-kaurene synthase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; NCED, 9-cis-epoxycarotenoid dioxygenase; NSY, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; Z-ISO, 15-cis-ζ-carotene isomerase; ZEP, zeaxanthin epoxidase" (Lu and Li, 2008).

All of these relations among altered genes suggest the multiple effects of different genes (normal functional or structural) underlying introgressed region of *S.pennellii* (Sub IL2-3/2-4) which result in different changes in genome expression pattern.

### 3.3.2 Could the transcription factor-Like genes, underlying introgressed region, be responsible for the changes in overall gene expression pattern?

In overall, four transcription factor (TFs) genes or TF-Like/dependent genes were identified in the introgressed region as follows: 1- Solyc02g081780.1 (B3 domain-containing protein), 2- Solyc02g082040.2 (MYB86-Like TF), 3- Solyc02g081670.1 (Cyclin-Like F-box), 4- Solyc02g082010.1 (Mitochondrial transcription termination).

1- Solyc02g081780.1 is a B3 domain-containing protein with regulation of transcription activity. Five major groups of genes containing the B3 domain are reported based on their similarities and domain structures; including proteins from the 1- *ABI3/VP1* (Suzuki et al., 1997), 2- *HSI* (High-level expression of sugar-inducible gene) (Tsukagoshi et al., 2005; Suzuki et al., 2007), 3- *ARF* (Auxin Response Factor) (Ulmasov et al., 1997), 4- *REM* (Reproductive Meristem) (Franco-Zorrilla et al., 2002), families and 5- *RAV* (Related to ABI3/VP1) (Kagaya et al., 1999). The *ABI3* and *HSI/VAL* families are involved in seed development and maturation (Tsukagoshi et al., 2005; Suzuki et al., 2007; Bäumlein et al., 1994; Stone et al., 2001; Koornneef et al., 1984). *RAV* genes class are not fully characterized, but some of them have been reported to be have a role in growth, development and flowering time (Hu et al., 2004; Alvarez et al., 2006; Castillejo and Pelaz, 2008). The major *REM* family did not represent any functional information available (Franco-Zorrilla et al., 2002), except the *VRN1* (*VERNALIZATION 1*), which acting in flowering promotion (Levy, 2002; Sung and Amasino, 2004). The best known family of B3 is the *ARF* family. This class regulates a range of responses to auxin and some other regulatory roles (Sessions et al., 1997; Mallory et al., 2005; Fahlgren et al., 2006; Guilfoyle and Hagen, 2007). Moreover, the B3 proteins functionally characterized from the *HSI*, *RAV*, *ABI3* and *ARF* families represent involvement in hormones, signalling pathways. For instance abscisic acid, auxin, brassinosteroid and gibberellin hormones (Romanel et al., 2009). The different effects of this TF family make this gene a good candidate gene, responsible for Q1968 line changes, but no data in RNA-seq was reported about this gene in any of stages of ripening.

2- Solyc02g082040.2 represent *MYB86-Like* transcription factor with DNA binding and regulation of transcription function. In plants the *MYB* superfamily is the most abundant group of transcription factors. MYB proteins include a *MYB* DNA-binding domain, which contains one to three helix-turn-helix sets of about 50 amino acids. Most of plants *MYB* TFs have two repeat of this domain (R2R3-*MYB*) (Yanhui et al.,

2006). *MYB* transcription factors play an important role in plant development and cell cycle regulation, disease resistance and abiotic stress tolerance, secondary metabolism, hormone signal transduction (Cominelli and Tonelli, 2009; Allan et al., 2008). Some of *R2R3-MYB* genes play role in regulating responses to environmental stresses such as drought, salt, and cold. (Agarwal et al., 2006; Yanhui et al., 2006) For instance *AtMYB96*, regulates drought stress response by ABA and auxin signals integrations (Seo et al., 2009). *GAMYB* class of transcriptional regulators affects gibberellins hormones (Gocal et al., 1999, 2001). Their role in secondary metabolite production like anthocyanin in blood orange also reported (Butelli et al., 2012). Considering the vast range of functions for this family, the *MYB86-Like* TF could be a very good candidate for the affective gene, but no reads reported for this gene in any of stages of ripening in either of lines.

3- Solyc02g081670.1 Cyclin-Like F-box with transcription factor binding activity and under ontology term of ubiquitin-protein ligase activity.

F-box proteins in eukaryotes, are an important component of SCF complex which belongs to ubiquitin E3 ligases family. This family of proteins are catalysing protein ubiquitination that result in balance between protein synthesis and degradation. In general, ubiquitin-proteasome system target and degrade non-functional and misfolded proteins which is the main major intracellular proteolysis machinery (Vierstra, 2009).

The E3 ubiquitin ligases consist of different family of proteins in plants. For instance in *Arabidopsis thaliana* more than 1,400 genes involve in this group (Gagne et al., 2002). Less than 5% of the F-box proteins have been functionally characterized, in *Arabidopsis thaliana*. F-box proteins take part in arrangement and regulation of different developmental processes such as phytohormone signalling (ethylene and gibberellins), defence responses, leaf senescence and branching, flower development and circadian rhythms (Woo et al., 2001; Stirnberg et al., 2007; Imaizumi and Kay, 2006; Chae et al., 2008; Dill et al., 2004; Ariizumi et al., 2011; Binder et al., 2007; Han et al., 2004; Kim and Delaney, 2002; Gou et al., 2009). It is shown a cyclin-Like F-box gene expressed in the all plant organs and tissues which actively dividing their cells. Also knockdown of cyclin-Like F-box proposed its possible role in cell cycle control as well. Therefore it is suggested that *F-box* genes can affect the cell cycle and cell proliferation and differentiation (Boycheva et al., 2015).

The mentioned roles of this group specially their effect on phytohormons like ethylene and gibberellins and cell cycle controls could correlate with many of the altered genes specially the most altered transcripts (see Table 3-11) which make the encoded gene a good candidate. But similar to two previous TF this candidate gene also did not present any reads in the RNA-seq data that make any prediction impossible and needs more investigations.

4- Solyc02g082010.1, Mitochondrial transcription termination (*mTERF*) factor family protein also observed among the genes of introgressed region and represented 1.5-fold increase in its expression at MG stage in Q1968 line compared to M82.

In the *Arabidopsis mTERF* family, 11 proteins of them were plastid-localized. It is shown that one plastidic *mTERF*, (*BELAYA SMERT* (*BSM*)), is required for embryogenesis. The genetic mosaics with the *BMS* mutation represent postembryonic phenotypes of distinct abnormalities in leaf development (Babiychuk et al., 2011). Mutant *BSM* cells are albino, are compromised in growth, and suffer defects in global plastidic gene expression (Babiychuk et al., 2011). Robles and colleagues suggested *TERF* genes influence on organelles gene expression, made them good candidate genes for participating in the coordination of nucleus-mediated mitochondria and chloroplast functions making them ideal candidates for the regulation of gene expression among organelles (Robles et al., 2012). Although these families of genes have a long way from detailed characterization, but their important roles, could make the Solyc02g082010.1 in the introgressed region a candidate gene.

### 3.3.3 Which gene is responsible for high lycopene Q1968 Sub-IL?

Q1968 is a robust high lycopene sub IL of *S.pennellii*. Pigment analysis in the 2 separate batches of Q1968 and M82 plants, grown in two different environment, confirmed this fact (nearly 2 times higher to M82 at ripe stage). It is difficult to judge about which gene is responsible for Q1968 line phenotypic alterations because a direct gene effecting carotenoid genes that affects directly on lycopene production could not be detected in the introgressed region. This means the phenotype of this sub-IL does not correlate with a clear gene that could be responsible for the phenotype.

Two types of effects could be considered for this line, the first one is the possible effect of some putative genes alongside each other for the generation of the Q1968 phenotype. This hypothesis seems rational; first because of closely related genes in

the region and in the changed overall expression patterns (see section 3.2.3.4.2 and 3.2.3.4.3) and secondly the vast expression changes in a variety of metabolic pathways not only in one stages of ripening but also in all of the stages seemingly needs the collaboration of other genes (for the explained relation among genes see section 3.3.1, 3.3.2). The second hypothesis is that one or some TFs are responsible for this new phenotype. The B3 domain (Solyc02g081780.1) and the *MYB86-Like* (Solyc02g082040.2) TFs with their different effects on different cellular mechanisms (detailed in section 3.3.2) are potential transcription factors, but absence of any data in RNA-seq data of these two TF, make it impossible to have a conclusion on them and also more supporting analysis is needed as well. It is worthy to analysis the level of the expression of these two TFs individually to see whether a difference will be detected in their expression rate or the RNA-seq data will be confirmed? The two other TFs also need more detailed analysis for verification, which can be done by our colleagues in future.

It is also plausible that correlation of one or some of presented TFs with some of functional/ structural genes resulting the vast differently expressed genes platform.

It is good to denote that it is not just one compound (Lycopene) that increased in Q1968 line, but also some other increased compounds detected that indicates the effect of differentially expressed genes on a vast range of metabolites other than just lycopene; such as amino acids. For instance, beta-alanine content was more than two times higher in most of stages of ripening in Q1968 compared to the control background M82 (Table 3-7). Some other examples from different groups like amino acids (glycine and isoleucine), organic acids (glyceric acid) and phosphates (inositol-6-phosphate) also mostly elevated. The fact that lycopene was not the sole metabolite that increased in Q1968 line but also other metabolites altered as well correlate with the proposed hypothesizes which effect of one TF or some TFs in the region or effect of some correlative genes could be responsible for altered pattern of expression in vast range of genes in the whole genome. Moreover, the alteration detected in carotenoid biosynthesis pathways was not that impressive to other differently expressed genes of other altered metabolites; such as inositol and glycine (22% of gene expression of carotenoid biosynthesis pathway altered in overall comparison of M82 and Q1968; This difference for inositol biosynthesis process and glycine metabolic process were higher to 80% and 38% respectively).

### 3.3.4 Which stages of ripening are affected more by the introgressed region in Q1968?

Some correlations were detected between the metabolite production/gene expression alterations and the stages of ripening. As previously shown in

, most of the changes in the expression of genes were detected at MG stages and this considerable effects resulted in some metabolite changes at MG stage to 3dpb. It is quite interesting to note that the rate of production of metabolites between MG to 3dpb is considerably higher among other stages (7dpb and 14dpb) in Q1968 to M82 (see Figure 3-11). These data also correlate with firmness factor data; These significant differences between fruit firmness factor just observed in breaker stage (Figure 3-5) which indicated Q1968 stayed firmer to M82 from MG to BR, but with a rapid rate (3 times more than M82) starts softening (from BR to 3dpb) and in 3dpb passed M82 softness level by 5 % Fff (Figure 3-5). Q1968 between BR and 3dpb decreased around 15 % of Fff while M82 decreased around 3 factors. Higher rate of metabolite production and rapid firmness changes of Q1968 correlate with impressive changes of gene expression at MG stage which conferring the main effect of Q1968 line at MG to 3dpb. These observations alongside with the most differently expressed genes at MG stage suggest that the responsible gene or genes have their main effect from MG stages to 3dpb on production of metabolites with a high rate and resulted in an initial alteration in start of softening. Predominantly the differences that appeared in the amount of metabolites at MG to 3dpb resulted the final increase of the metabolites at later stages such as 7dpb and 14dpb of Q1968 line; in other words this initial difference in the level of metabolite at MG and 3dpb of Q1968 keep its distance with M82 in 7dpb and 14dpb as well (see Figure 3-11). It can be conducted that the overall transcript changes at MG stage resulted the final metabolite changes in Q1968 line.

The responsible gene/genes in the introgressed region also affect the master ripening TFs (*CNR, RIN, NOR* and *NR*) mainly at MG stage, which could propose effect of the responsible gene by other mediated TFs and gene that needs further investigations.

Considering the significant changes in some important pathways effecting DNA replication, cell division, cell elongation such as gibberellins and carbon fixation alongside with some chlorophyll changes, it is recommended to have more investigations on cellular and hormonal changes at immature green (IMG) and mature green stages to see the probable alterations. For instance, auxin and gibberellins

hormonal level detection, cell numbers, size and plastid assessments and etc. to have a deeper cellular, hormonal and metabolite insight about the changes at IMG and MG stages and making more robust conclusion about Q1968 line at MG stage.

As illustrated in Figure 3-11 from 3dpb to 7dpb the rate of changes in metabolite production in Q1968 and M82 is nearly the same. Another considerable observation was about this fact that most of considerable changes in metabolite levels comes to view from 7dpb onward, and also the rate of accumulation of metabolites again increased between 7dpb and 14dpb in Q1968 (see Figure 3-11). This time the difference in rate of production is not as impressive as that between MG to 3dpb. This means that there are some important points to be addressed about storage properties of this line. Adding to this observation the statistical report about the number of changed genes indicates that the number of downregulated genes of Q1968 to M82 is nearly doubled at 7dpb in comparison to 3dpb (see Table 3-8), making this stage the second most affected stage by the introgressed region and half of the downregulated genes at ripe stage are overlapping with downregulated genes at MG stage (see Figure 3-8). The effect of these changes still needs to be investigated, but at metabolite level the rate of increase is on going more than M82. The matter could be about keeping the transcripts active and continuing the production and accumulation of metabolites within cells in Q1968 line to M82 or some other mechanism. This hypothesis also correlates with the observation in RNA extraction that at ripe stage with equal quantity of starting materials always Q1968 line showed more distinct bands on the electrophoresis gel and also better fruit quality observed for Q1968 line (data not shown).

Overall, as shown in Figure 3-12 two peaks of alterations in the transcription pattern and subsequently metabolites level were detected in Q1968 to M82. The first changes in the transcripts was at MG stage (subsequently the results on metabolite production rate comes to view between MG to 3dpb). The second alteration in differently expressed genes occurred at 7dpb stage (the result on metabolite production rate observed in 7dpb to 14dpb). The schematic rate of changes illustrated in Figure 3-12.

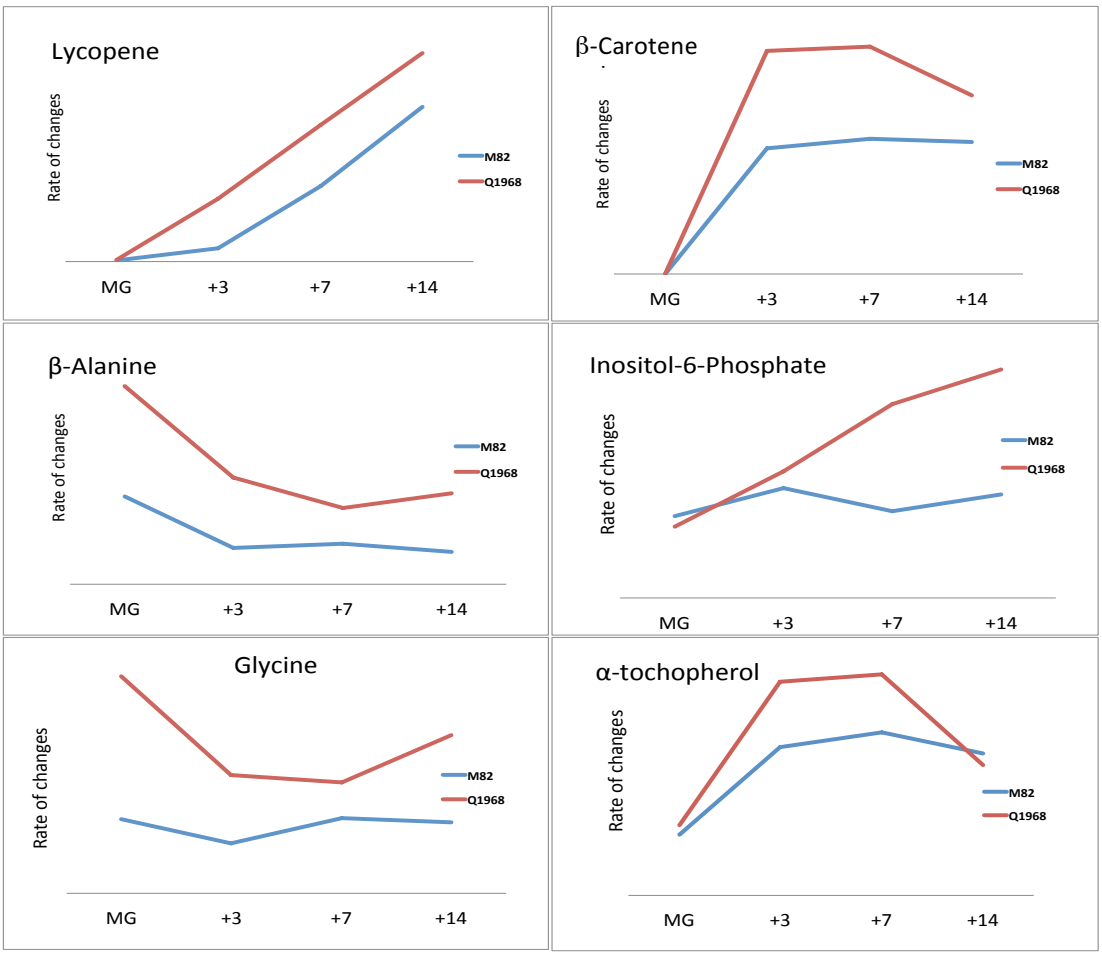



Figure 3-11 Rate of production of metabolites during stages of ripening.

The metabolite data of some compounds from UPLC (pigment analysis) and GC analysis (metabolomics data) have plotted during stages of ripening in two lines of M82 (blue line) and Q1968 (red line). MG, +3, +7 and +14 represent Mature green; 3 days post breaker (dpb), 7 dpb and 14 dpb respectively. There is 5 to 6 days between MG and +3 stages of ripening, 4 days between +3 and +7 and 7 days between +7 and +14. This figure represent the rate of some metabolite changes during stages of ripening; for detailed data of amount of metabolites and their significant differences see sections 3.2.3.2 and 3.2.3.3.

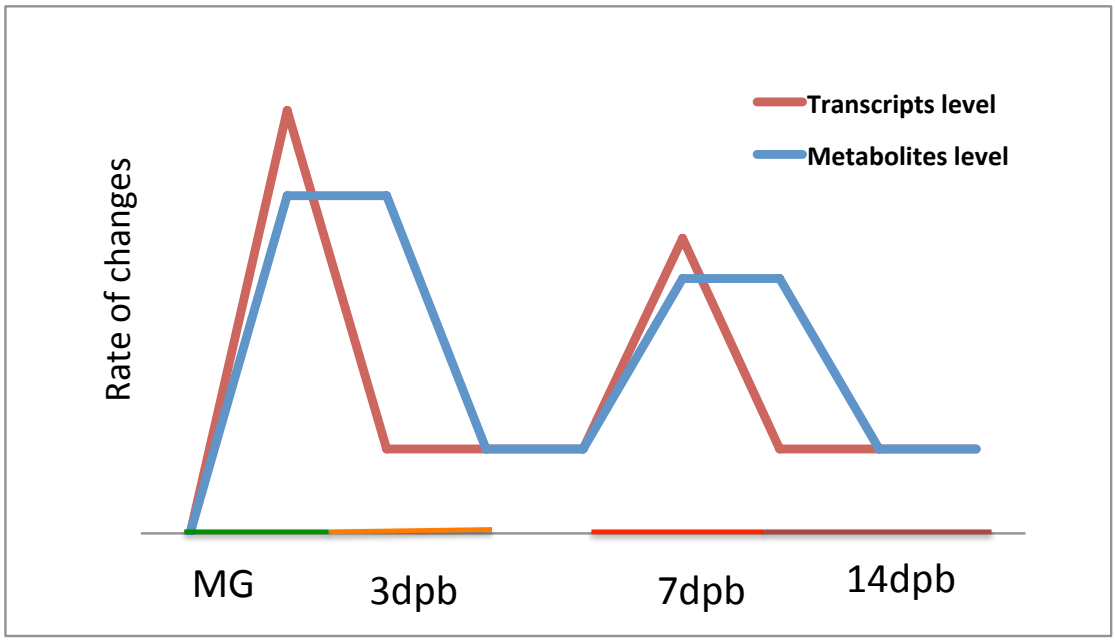



Figure 3-12 Schematic changes in rate of transcripts and consequently metabolites in Q1968 line to its wild type control M82.

The absolute overall changes of Q1968 to M82 at different stages of ripening are drown based on statistical reported alterations in RNA-seq data for transcripts level (red line) and for metabolites level from the extracted rate of changes of some metabolites (blue line) (Figure 3-11).

# CHAPTER 4: COMBINING KETOCAROTENOID SYNTHESIS WITH PUTATIVE TRANSCRIPTIONAL REGULATORS

## 4.1 Introduction

A diverse variety of strategies have been used to increase carotenoid formation and accumulation in plants. These include the metabolic engineering of carotenoid pathway (Enfissi et al., 2005; Römer et al., 2000; Wurbs et al., 2007), either through the expression of heterologous genes or the expression/down-regulation of transcription factors influencing carotenoid production (Broun and Somerville, 2001). The use of genetic crossing is a traditional way of combining genetic regions conferring traits of interest. Some new genotypes derived from genetic crosses have been analysed in this study. The parents used for crossing are described here briefly. Tomato line of CrtZW was used as one of the parents, containing the bacterial genes of β-carotene ketolase/hydroxylase (*CrtZW*). The *CrtZ* (*β-carotene hydroxylase*) and *CrtW* (*β-carotene ketolase*) genes were originated from a marine bacteria *Brevundimonas sp* strain SD212 with specific plant codon (Hasunuma et al., 2008). These biosynthetic genes provide the plant with the biosynthetic potential to produce of non-endogenous ketocarotenoids. These ketocarotenoids in question are of high vale and used as supplements in animal and aquaculture feeds. They are also potent antioxidants, with health-promoting properties (Breithaupt, 2007; Yuan et al., 2011).

DET1 and APRR2 tomato lines, which consist of down regulated *DET1* (Davuluri et al., 2004) and overexpressed *APRR2* genes (Pan et al., 2013) respectively, were used as the other parent genotypes in the crossing program. *DET*1 (*DE-ETIOLATED*1) gene contributes in suppression of the light responses (Davuluri et al., 2004). A fruit-specific down-regulation of this gene results in the coordinated increase of photosynthesis/core metabolism, and subsequently the elevation of chloroplast area per cell, which in turn manifests itself in the increased primary and secondary metabolites (Enfissi et al., 2010). Increased chlorophyll content in mature green stage and carotenoids at ripe stages is significant in this line (Enfissi et al., 2010; Davuluri et al., 2004). Genetic cross between DET1 and CrtZW lines were performed in order to optimise ketocarotenoid production. Another transgenic line overexpressing the tomato *APRR2-Like* gene (ARABIDOPSIS PSEUDO RESPONSE REGULATOR2-LIKE) was used to cross with CrtZW. Overexpression of APRR2 gene increased plastid area, number, and pigment content. It also enhanced the levels of chlorophyll in immature fruits and carotenoids level in ripe fruits (Pan et al., 2013).

In this chapter two lines of CrtZW.DET1 and CrtZW.APRR2, produced by genetic crossing, have been studied.

The strategy used in this study is coordinating expression of *CrtZW* with effective transcription factors to improve the production of ketocarotenoids in plants.

## 4.2 Results: Characterisation of genotypes containing combinations of ketocarotenoid biosynthetic genes and transcription factors (*DET1* and *APRR2*)

### 4.2.1 CRTZW.DET1 phenotype characterisation

Tomato seeds from the genotype containing the stacked transgenes encoding the β-carotene ketolase/hydroxylase gene (*CrtZW*) under the control of the 35S promoter (Enfissi et al, unpublished) were crossed with the *DE-ETIOLATED1* (*DET1*) down-regulated line under the control of the TFM7 promoter. The cross pollination of these lines was performed by Dr Enfissi. Phenotypic characterisation of the progeny has been described in the subsequent section. The CrtZW and DET1 lines have been grown for many (more than ten) generation and they kept their transformed characteristics over ten generations. This study made the main crosses between three differently transformed pairs of CrtZW and DET1 lines and choose the best lines of their first generation for further experiments.

#### 4.2.1.1 Characterisation of the observed phenotypes

Seeds from F1 generation were sowed alongside with parental lines of CrtZW (MM), DET1 (T56) and wild type backgrounds of T56 and Money Maker (MM). Figure 4-1 illustrates the phenotypic differences in fruit size and colour of these lines. Tomato lines of CrtZW and its wild type had medium fruit size (3-4 cm in diameter) while the wild type T56 background had large size tomato fruits (7-9 cm in diameter) and DET1 had the same approximate size as T56. In terms of fruit size, crossed line of CrtZW.DET1 was more similar to CrtZW with fruit diameter of 4-5 cm (Figure 4-1).

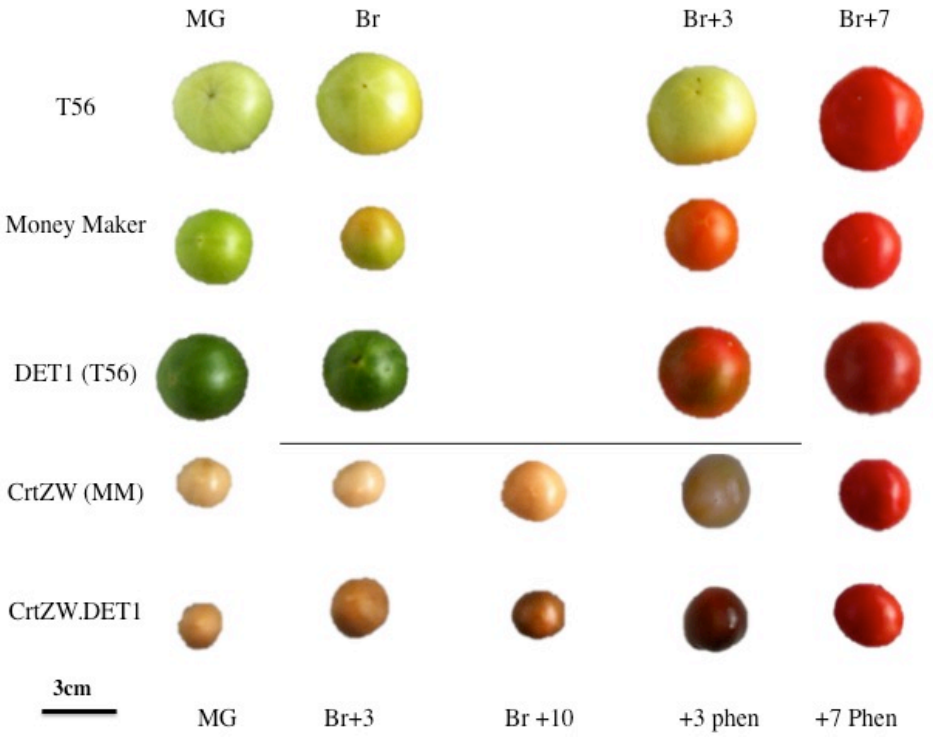



Figure 4-1 Phenotypic differences in fruit size and colour of crossed F1 CrtZW.DET1 and its parental lines and wild type backgrounds.

The first three lines indicate fruit size and colour in Mature green, Breaker (Br), 3 days post breaker and 7 days post breaker in T56, Money Maker (MM) and DET1 (with background of T56) respectively. The fourth and fifth line show fruit size and colour in CrtZW (with background of MM) and CrtZW.DET1 respectively in following steps: Mature green (MG), Breaker (Br), 10 days post breaker (Br+10), 19 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in normal ripening pattern (+3 Phen); and 25 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage in normal ripening pattern (+7 Phen). The scale bar indicates 3 cm.

F2 generation seeds were chosen from the best lines of F1 generation based on pigment characterisation; which lines with higher quantity of chlorophylls, total carotenoids and ketocarotenoids were chosen for next generation growing and further analysis. All plants were investigated for phenotypic differences. Leaf, steam and flower colour of CrtZW.DET1 line was similar to its CrtZW parent as expected in due of fruit specific TFM7 promoter of *DET1* gene, while the fruit colour was a combination of pink CrtZW fruits and dark green of DET1 lines in MG stage (see Figure 3-4) and deep red in ripe stage (see Figure 4-3). The fruit size was also similar to CrtZW. Other three derived lines as controls also were grown for further experiments, including Control (T56.MM), CrtZW (T56.MM) and DET1 (T56.MM) lines (Figure 4-3).


Figure 4-2 A comparison of phenotypic differences between the flowers and fruits colour in the control, CrtZW, DET1 and CrtZW.DET1 genotypes.

A. Flower colour of Control and DET1 lines (the picture was taken from DET1 line), B. Flower colour of CrtZW and CrtZW.DET1 lines (the picture was taken from CrtZW line), C. Control line (T56.MM) fruit colour, D. CrtZW fruit colour, E. DET1 fruit colour, F. CrtZW.DET1 fruit colour.

It was previously observed that the ripening process was delayed in CrtZW lines. Typically, it was observed that there is around 22 days between breaker to ripe stage, which in wild type background of MM it is 7 days. (Enfissi et al., unpublished), in CrtZW.DET1 a delay of 23-25 days was observed from breaker to ripe fruits compared to control lines (MM & T56 & MM.T56). Figure 4-3 shows the fruits collected in different stages of ripening in CrtZW.DET1 and its derived lines of Control (T56.MM), CrtZW and DET1.

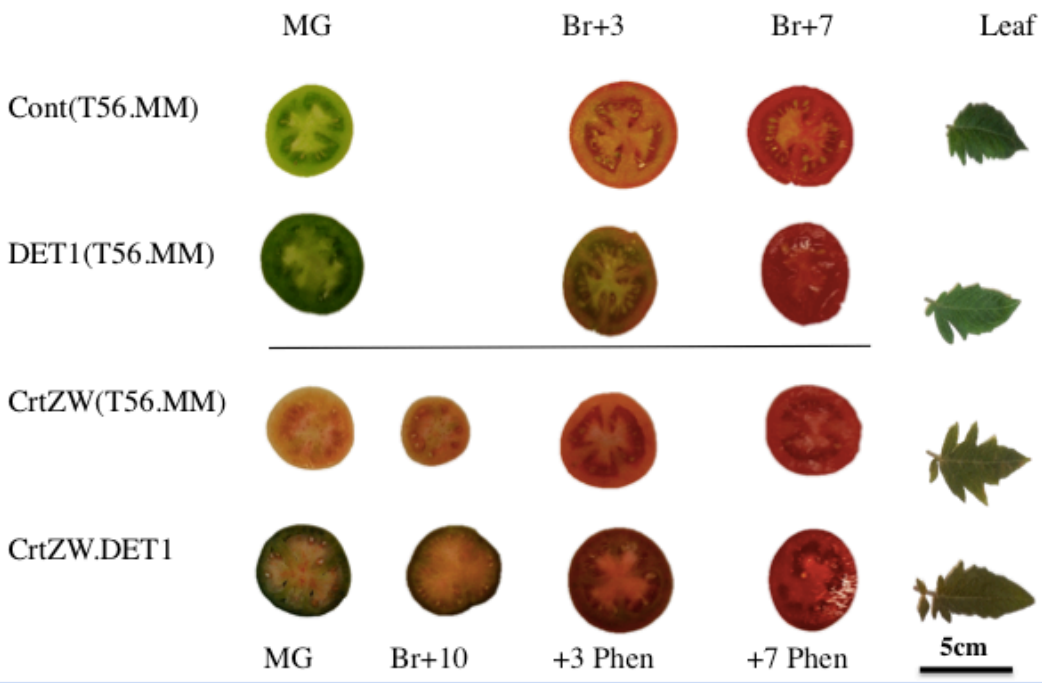



Figure 4-3 Leaf and fruits colour in developmental stages of ripening in F2 CrtZW.DET1 and its derived lines.

The first and second rows indicate the leaf and fruit colour in Mature green, 3 days post breaker, 7 days post breaker and leaves in the derived F2 Controls (T56.MM) and DET1 (T56.MM) respectively. The third and fourth rows show the leaf colour and the colour of fruit cuts in F2 generation of CrtZW (T56.MM) and CrtZW.DET1 respectively in the following steps: Mature green (MG), 10 days post breaker (Br+10), 19 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in normal ripening pattern (+3 Phen); 25 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage which is observed in a normal ripening pattern (+7 Phen); and the colour of their leaves. The scale bar indicates 5 cm.

#### 4.2.1.2 Firmness characterisation

Measurement of the firmness factor in the fruit from these genotypes also corroborated the delay in ripening. The normal control line of (T56.MM) ripe in 7 days had a firmness factor of around 70 % Fff. The firmness factor of DET1 line in 7 days post breaker reach to 80 % Fff, which produce firmer fruits to normal tomatoes (Figure 4-4). But the main difference for delay in ripening was observed in lines containing *CrtZW* gene. In addition to firmness factor, the colour change was also confirmed the delay in ripening. The two lines of CrtZW and CrtZW.DET1 ripe 22 and 25 days after breaker point respectively (Figure 4-4), which showed 15 and 18 days delay in ripening to control line.

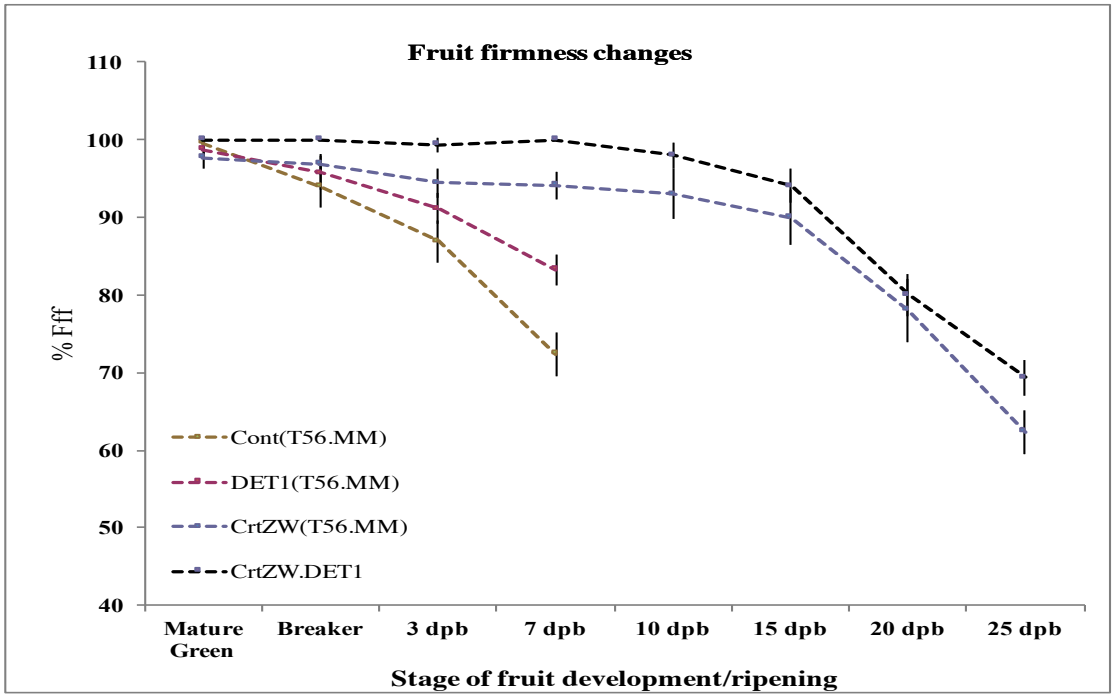



Figure 4-4 Percentage of Fruit firmness factor (Fff) changes during fruit developmental stages and ripening for CrtZW.DET1 lines and the derived control, CrtZW and DET1 lines.

Firmness measurement performed in different stages of ripening by measuring the firmness for at least 3 tomato fruit with 3 measurements for each fruit at each stage of ripening. Stages of ripening defined as Mature green, breaker and 3 to 25 days post breaker (dpb). Error bar indicates the ± Standard Deviation (S.D) of measurements with n=9 for each point. For more details see section 2.1.2.

#### 4.2.1.3 Designation the stages of ripening

These phenotypic differences in various stages of ripening result in definition of new stages of ripening for these two lines (CrtZW and CrtZW.DET1) for comparison and discussion of results with wild type and control lines that follow the normal 7 days post breaker period of ripening (see section 2.1.3.2). Stages of ripening and their comparison to the normal staging of tomato ripening is illustrates in Table 4-1. Based on the colour change and firmness factor equivalent steps were defined for delayed ripening lines as follows: 19 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in a normal ripening pattern defined as +3 Phen and 25 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage in normal ripening pattern, designated as +7 Phen (see Table 4-1)

Table 4-1 Designation of stages of ripening in lines with normal ripening pattern and lines with delayed ripening pattern.

Stages of ripening are defined as Mature green (MG), Breaker (Br), 10 days post breaker in delayed ripening lines (Br+10), 19 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in normal ripening pattern (+3 Phen) and 25 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage in normal ripening pattern (+7 Phen).

| | | | | | |
|---|---|---|---|---|---|
| Normal ripening | MG | Br | | Br+3 | Br+7 |
| Altered ripening | MG | Br | Br+10 | +3 Phen (Br+19) | +7 Phen (Br+25) |

#### 4.2.1.4 Confirmation of existence of transgenes

The existence of *CtrZW* was confirmed by normal PCR (see sections 2.3.2.2 and 2.3.2.3) of an amplicon of *CrtW* and *CrtZ* genes (see Figure 4-5) and the presence of a down-regulated *DET1* transgene confirmed by detecting changes in transcript level of the *DET1* gene. As the transcript level of *DET1* gene was significantly lower compared to its control line conferring the down-regulated *DET1* gene in CrtZW.DET1 line. More details about the transcript level of DET1 were explained in expression section (see section.4.4).

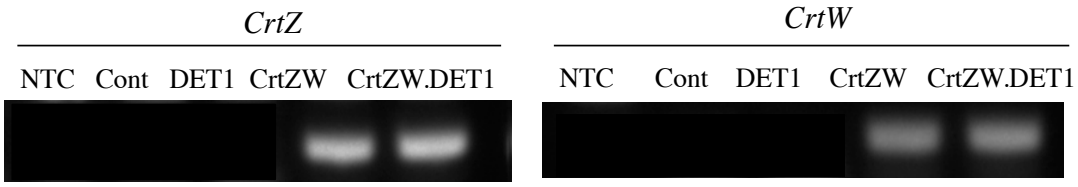



Figure 4-5 PCR confirmation of the presence of *CrtZ* and *CrtW* genes in the CrtZW.DET1 line and its derived lines.

Amplification of an amplicon of *CrtZ* and *CrtW* genes was performed by PCR on the DNA pool sample of 9 biological replicates of the CrtZW.DET1 and its derived lines separately. Gel results visualised under UV light. NTC: non-template control; Cont: Derived Control line from genetic cross (T56.MM). CrtZW: Derived CrtZW line from genetic cross (T56.MM), DET1: Derived DET1 line from genetic cross (T56.MM) and CrtZW.DET1 line.

In summary the genetic crossing of the CrtZW and DET1 genotypes has produced new genotypes of CrtZW.DET1, with new phenotypic and developmental characteristics. Further characterisation of these lines was undertaken through further

experimentation to see weather the aim of this objective which was improvement of ketocarotenoids and other high value compounds attained or not.

### 4.2.2 CRTZW.APRR2 phenotype characterisation

Tomato variety seeds containing transgenes of (the β-carotene ketolase/hydroxylase gene (*CrtZW*) (Enfissi et al., unpublished) and *ARABIDOPSIS PSEUDO RESPONSE REGULATOR2-LIKE* (*APRR2-Like* gene) (Pan et al., 2013) both under 35S promoter control, produced by genetic crossing, were used for the characterisation of the new genotype of CrtZW.APRR2.

#### 4.2.2.1 Phenotypic characterisation

Phenotypic differences in this line were significant resulting from the crossing between MicrotomMicrotom background of APRR2 line with CrtZW line. The MicrotomMicrotom background height was around 20-30 cm while the CrtZW (MM background) plant height was around 2 m. The height of the crossed plants was in between Microtom and normal MM variety, about 0.5-1 m length (see Figure 4-6) which is in result of combination of two genotype and production of new characteristics in their offspring (it seems neither of them were dominant and an intermediate height was produced).

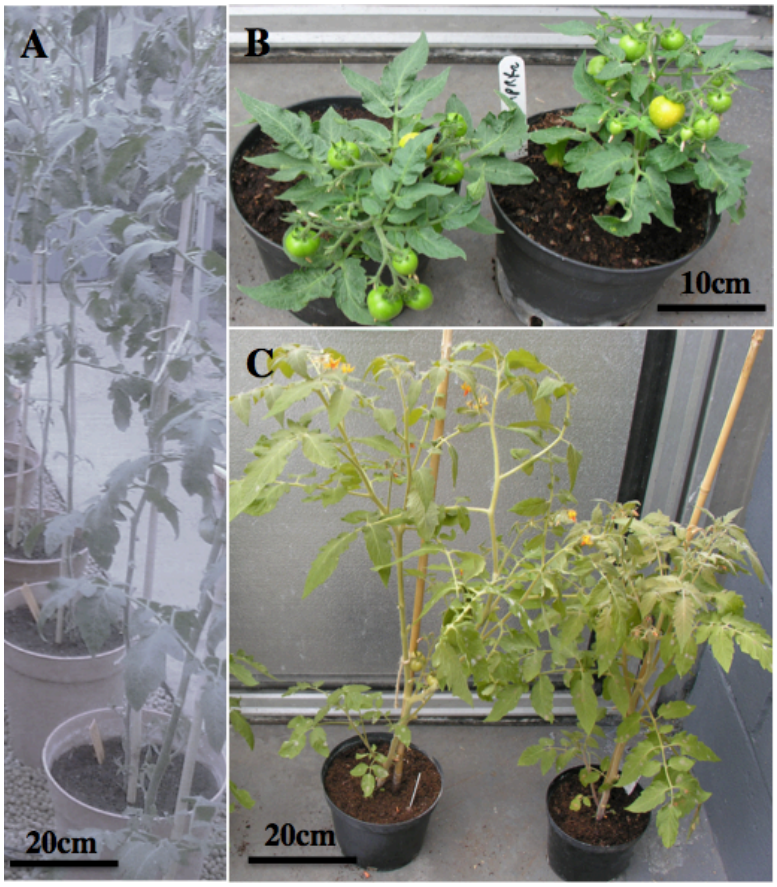



Figure 4-6 Plant size change in crossed line of CrtZW.APRR2.

A: Parental CrtZW (MM) line with plant size of about 2 m, B: Parental APRR2 (Mic) line with plant size of about 20-30 cm. C: Crossed line of CrtZW.APRR2 with plant size of 0.5-1 m length.

As shown in Figure 4-7 flower colour of CrtZW.APRR2 and CrtZW parent is the same while the stem and fruit colour of CrtZW.APRR2 was a combination of CrtZW pink fruits and green colour of APRR2 fruits in MG stage. Leaf colour in CrtZW.APRR2 was darker in comparison to CrtZW, which could be due to the 35S promoter controlling the *APRR2* gene (see Figure 4-8). The fruit size of CrtZW.APRR2 was 2-3 cm in diameter, between two parental varieties (Figure 4-8). Considering fruit firmness and colour changes (see Figure 4-8 & Figure 4-9), in CrtZW (Mic.MM) and CrtZW.APRR2 the ripening delayed about 14 days between breaker to ripe stage in comparison to normal ripening lines.

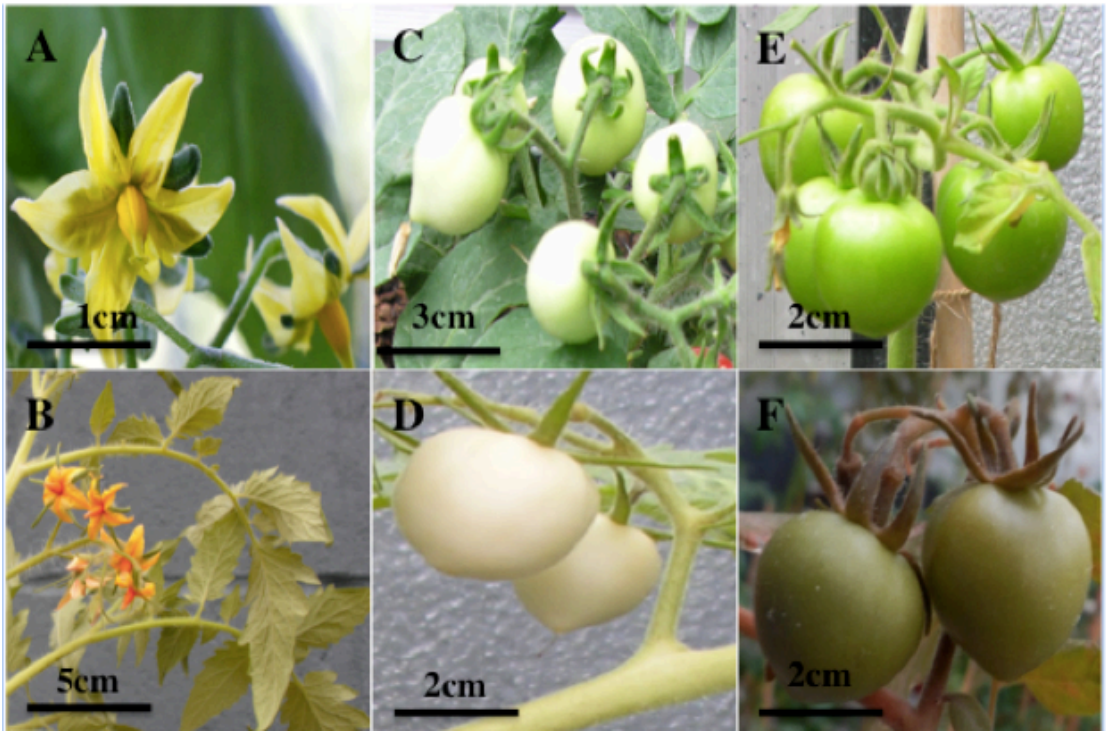



Figure 4-7 Phenotypic differences in flower and fruits colour in Control, CrtZW, APRR2 and CrtZW.APRR2.

A. Flower colour of Control and APRR2 lines (the picture was taken from APRR2 line), B. Flower colour of CrtZW and CrtZW.APRR2 lines (the picture was taken from CrtZW line), C. Frit colour of Control (Mic.MM) line, D. CrtZW fruit colour, E. APRR2 fruit colour, F. CrtZW. APRR2 fruit colour.

Figure 4-8 illustrates the colours and sizes of fruit cuts collected in different stages of ripening in CrtZW.APRR2 and its derived lines of Cont (Mic.MM), CrtZW (Mic.MM) and APRR2 (Mic.MM).

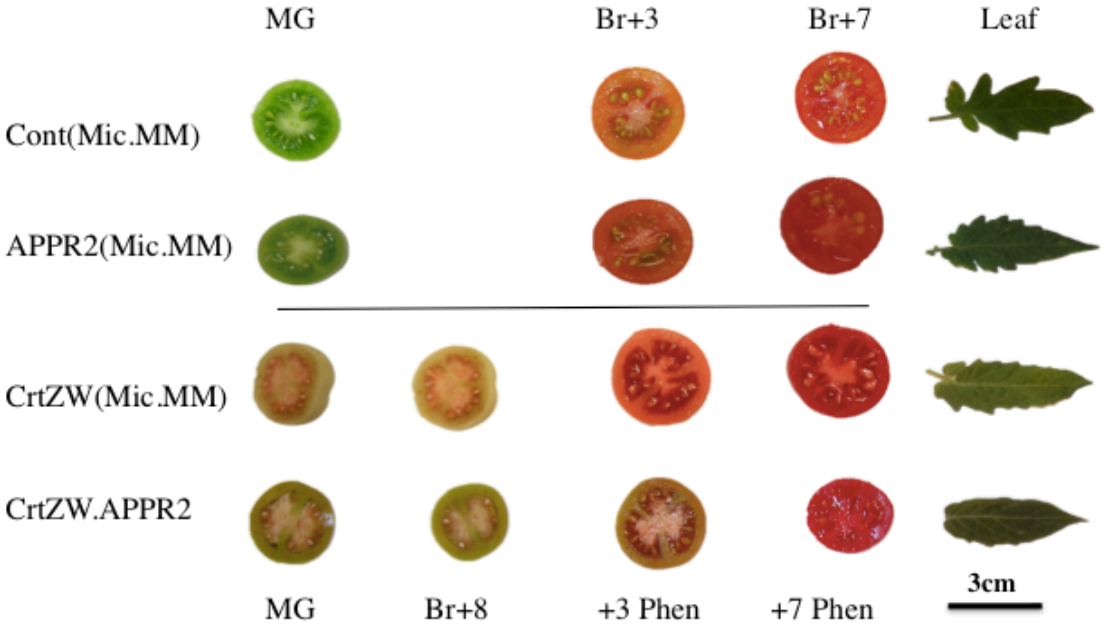



Figure 4-8 Leaf and fruits colour in developmental stages of ripening in CrtZW.APRR2 and its derived lines.

The first and second lines indicate the leaf and fruit cuts colour in Mature green, 3 days post breaker and 7 days post breaker in Control (Mic.MM) and APRR2 (Mic.MM) respectively. The third and fourth lines show the leaf colour and the colour of fruit cuts in CrtZW (Mic.MM) and CrtZW.APRR2 respectively in following steps: Mature green (MG), 8 days post breaker (Br+8), 10-12 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in normal ripening pattern (+3 Phen) and 14-15 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage in normal ripening pattern (+7 Phen). The scale bar indicates 3 cm.

#### 4.2.2.2 Firmness characterisation

The firmness factor measurement matched the observation that ripening was delayed. As Figure 4-9 illustrates the wild type and normal varieties of tomato ripen 7 days after breaker and the firmness of ripe fruits was normally around 65-75 % Fff. The parental APRR2 (MicrotomMicrotom) and also derived APRR2 (MicrotomMicrotom, MM) in F2 generation followed the normal pattern of ripening. But the lines containing *CrtZW* gene had another pattern for ripening. Their colour changes and also the firmness factor data showed delay in ripening. The two lines of CrtZW (Mic.MM) and CrtZW.APRR2 ripe around 15 days after breaker stage (see Figure 4-9). These varieties ripe 7-8 days later after normal tomatoes.

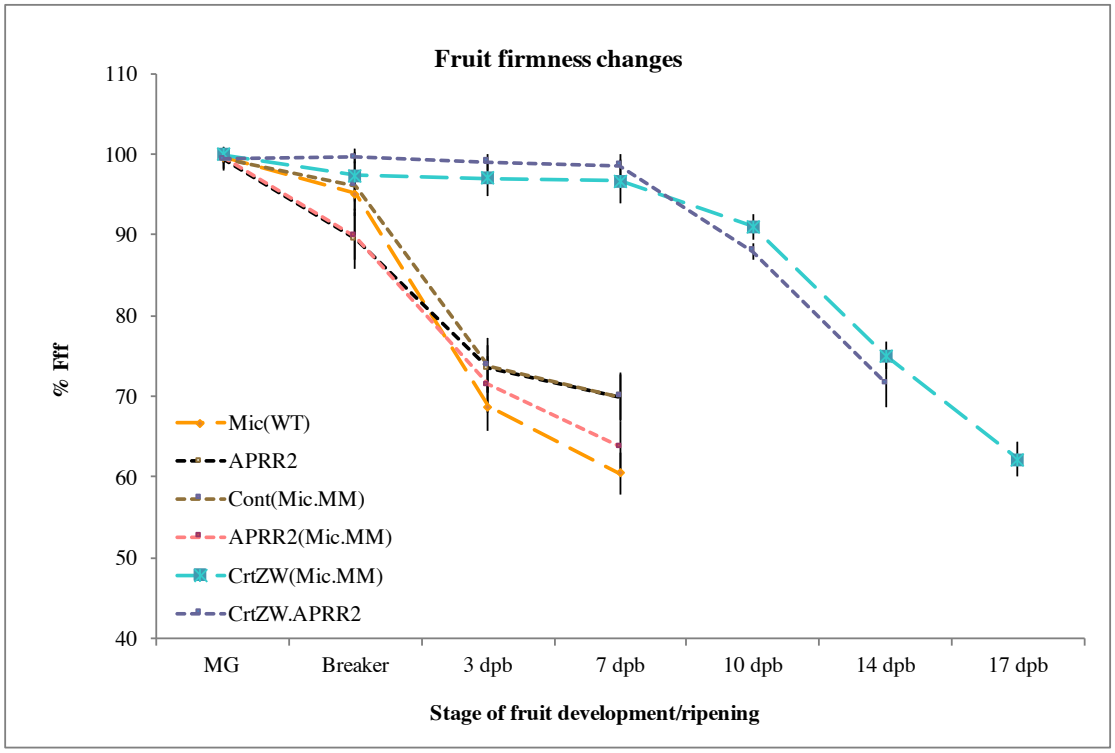



Figure 4-9 Percentage of Fruit firmness factor (Fff) changes during fruit developmental stages and ripening for CrtZW.APRR2 lines and its derived lines: Control (Mic.MM), CrtZW (Mic.MM) and APRR2 (Mic.MM) lines. APRR2: The parental line of APRR2 with MicrotomMicrotom background. Mic (WT): The wild type Microtom variety.

Firmness measurement performed in different stages of ripening by measuring the firmness for at least 4 tomato fruit with 3 measurements for each fruit at each stage. Stages of ripening defined as Mature green, breaker and 3 to 25 days post breaker (dpb). Error bar indicates the ± Standard Deviation (S.D) of measurements with n=12 for each point. For more details see section 2.1.2

#### 4.2.2.3 Defining the stages of ripening

In a similar manner to CrtZW.DET1 line, these differences in phenotypic stages of ripening leaded to defining new stages of ripening for the two lines of CrtZW and CrtZW.APRR2 for comparing and discussing the results with control lines which follow the normal 7 days post breaker period of ripening (section 2.1.3.2). Schematic steps of ripening and their comparison to normal pattern of tomato ripening are illustrated in Table 4-2. Based on colour change and firmness factor equivalent steps were defined for delayed ripening lines as follow: 10-12 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in normal ripening pattern (+3 Phen) and 14-15 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage in normal ripening pattern (+7 Phen).

Table 4-2 Definition of stages of ripening in lines with normal ripening pattern and lines with delayed ripening pattern.

Stages of ripening are defined as Mature green (MG), Breaker (Br), 8 days post breaker in delayed ripening lines (Br+8), 10-12 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +3 stage in normal ripening pattern (+3 Phen) and 14-15 days post breaker in delayed ripening lines which phenotypically is equivalent to breaker +7 stage in normal ripening pattern (+7 Phen).

| | | | | | |
|---|---|---|---|---|---|
| Normal ripening | MG | Br | | Br+3 | Br+7 |
| Altered ripening | MG | Br | Br+8 | +3 Phen (Br+10-12) | +7 Phen (Br+14-15) |

#### 4.2.2.4 Confirmation of existence of transgenes

The presence of CrtZW genes was confirmed by normal PCR of an amplicon of *CrtZ* and *CrtW* genes (see Figure 4-10) and existence of overexpressed transgene of *APRR2* was confirmed by increased *APRR2* transcripts in the over expressed APRR2 line in comparison to control. More details described in expression section (see section 4.4).

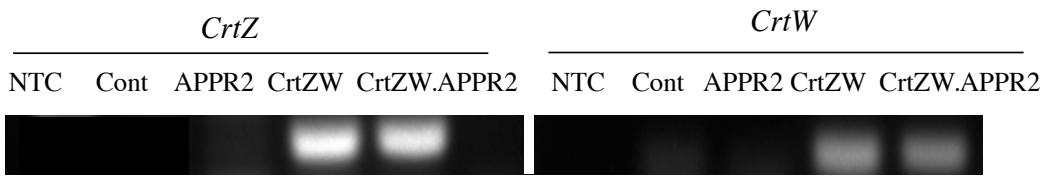



Figure 4-10 PCR confirmation of the presence of *CrtZ* and *CrtW* genes in the CrtZW.APRR2 line and its derived lines.

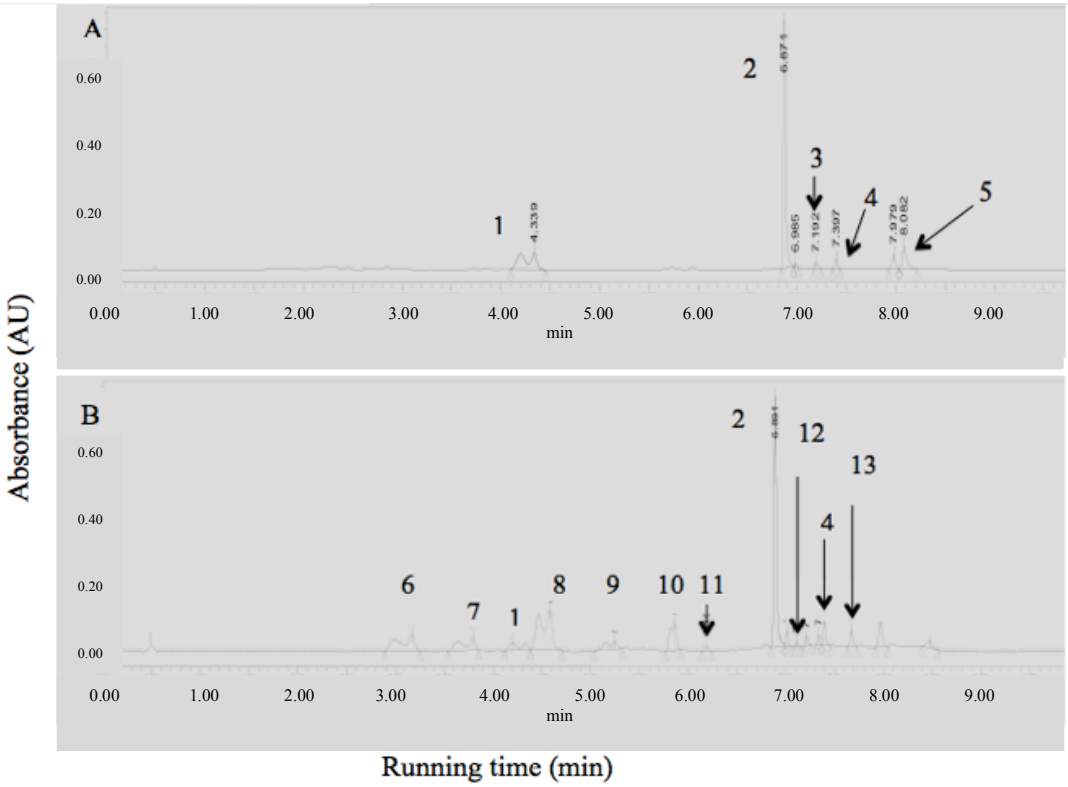
Amplification of an amplicon of *CrtZ* and *CrtW* genes was performed by PCR on the DNA pool sample of 9 biological replicates of the CrtZW.APRR2 and its derived lines. Gel results visualised under UV light. NTC: non-template control; Cont: Derived Control line from genetic cross (Mic.MM). CrtZW: Derived CrtZW line from genetic cross (Mic.MM), APRR2: Derived APRR2 line from genetic cross (Mic.MM) and CrtZW.APRR2 line.

Overall, genetic crossing between CrtZW and APRR2 produced the new line of CrtZW. APRR2 with unique phenotypic characteristics. Although the phenotypic differences in CrtZW.APRR2 were not as prominent as CrtZW.DET1, Further characterisation of these lines was performed through subsequent experimentation (see sections 4.2.3, 4.2.4, 4.3, 4.4, 4.5 for results and section 4.7.2 for conclusion).

### 4.2.3 Assessment of carotenoid profiles in tomato fruits during fruit development and ripening

#### 4.2.3.1 Assessment of ketocarotenoids based on UPLC-PDA derived spectrum

For the investigation of ketocarotenoids changes along side with other pigments, chromatographic analysis of ketocarotenoids and other pigments was carried out on the lines containing ketocarotenoids (CrtZW, CrtZW.DET1) and their appropriate comparators (Control, DET1). Extracted fruit samples were analysed under Ultra and High Performance Liquid Chromatography (UPLC-PDA) to have a better understanding about the level of ketocarotenoids and other pigments for detecting pigment profile changes. The UPLC method is described in section 2.2.3. Figure 4-11 illustrates sample chromatogram profiles of ketocarotenoids and some other pigments and their spectral characteristics of CrtZW line and its Control (T56.MM) line. Based on the program, which was used for UPLC, the retention time of eluted pigments varies. All of ketocarotenoids were detectable in all stages of fruits ripening and also in leaves at 450 nm. See Figure 4-11 for more details about the retention time, exact spectra for each ketocarotenoid and related peak on chromatographs. For the detectable wavelength and exact spectra of other carotenoids, α-tocopherol and chlorophylls and their related calibration equations refer to appendix 1 Figure A 7-1 and appendix 2 Figure A 8-1.




| Name | Retention time (min) | Spectra $\lambda_{max}$ (nm) | Peak number |
|---|---|---|---|
| Chlorophyll a | 7.2 | 429.8 | 3 |
| Astaxanthin | 3.17 | 479.0 | 6 |
| Adonixanthin | 3.8 | 474 | 7 |
| Lutein | 4.34 | 448, 476 | 1 |
| Adonirubin (Phenicoxanthin) | 4.59 | 478.4 | 8 |
| Unknown1 | 5.25 | 453.0 | 9 |
| Canthaxanthin | 5.88 | 476 | 10 |
| Echineone | 6.19 | 467 | 11 |
| Chlorophyll b | 6.88 | 456.6 | 2 |
| Unknown 2 | 7.12 | 478.0 | 12 |
| Unknown 3 | 7.69 | 466.0 | 13 |
| Pheophytin b | 7.4 | 434.7 | 4 |
| β-Carotene | 8.08 | 454.1, 479.6 | 5 |

Figure 4-11 Chromatographic profiles and spectral characteristics of Ketocarotenoids and some other pigments in Control (T56.MM) and CrtZW line analysed by UPLC.

A. Fruit chromatograph of Control (T56.MM) in mature green stage at 450 nm, B. Fruit chromatograph of M82 in mature green stage at 450 nm. The table illustrates the spectral characteristics of pigments (recorded from 250 to 600 nm) using UPLC, retention time and the spectra for each pigment is shown and peak numbers are indicated on chromatographs.

#### 4.2.3.2 Best line selection through first generation pigment profile for CrtZW.DET1 and its parental and background controls lines.

Best lines selection through first generation pigment profile of 11 CrtZW.DET1 plants were performed. UPLC-PDA analysis was performed as described in section 2.2.3. Pigment profile of MG fruits, 3 dpb and 7 dpb fruits were carried out for 11 CrtZW.DET1 lines, the parental lines (CrtZW & DET1) and the two backgrounds varieties of parental lines (T56 & MM). The ketocarotenoids and other pigment content for 11 CrtZW.DET1 lines to its CrtZW comparator is shown in Table 4-3, Table 4-4 and Table 4-5 for MG, +3 Phen and +7 Phen (ripe) stages respectively.

For an overall view about the changes in CrtZW.DET1 to its parental and wild type backgrounds, the pigment changes of the two best selected lines of CrtZW.DET1 and its parental and background lines at MG and ripe stages of ripening is shown in Table 4-6 and Table 4-7; Also for a better visual prospect, the same data for MG and ripe stage is illustrated in Figure 4-12. For selecting the best line, the ketocarotenoids content changes were considered at all stages of ripening. In addition, chlorophylls levels at MG stage and lycopene content at ripe stage were the second criterion for best line selection. The highest elevation in ketocarotenoids, lycopene and chlorophyll content were observed in line 2 and 4. The chlorophyll a & b contents were increased at MG stage for these two lines of CrtZW.DET1 around 3 & 4.5 times to CrtZW respectively. Some of the ketocarotenoids were elevated at the MG stage while at ripe stage most of them increased. Adonirubin increased around 3.5 fold in line 2 & 4 at MG stage, which reached to 5 and 4 fold at ripe stage for line 2 & 4 respectively (Table 4-3 and Table 4-5). Astaxanthin as another important ketocarotenoid indicate a rise of around 4 and 2.5 fold at ripe stage for line 2 & 4 respectively; this increase at +3 Phen was 1.5 and 2.5 times for line 2 & 4 respectively (Table 4-4 and Table 4-5). Lycopene, which is the most important indigenous carotenoids in tomato fruits, with 2 fold increase showed the highest improve in line 2 and in line 4 increased by 1.5 fold to CrtZW. Therefore, based on the best improved level of pigments in different stages of ripening, the two lines of CrtZW.DET1 (line 2 and line4) were chosen for subsequent steps of experiments for F2 generation (see the overall view in Figure 4-12). The seeds of these lines sowed for growing the best F2 plants of CrtZW.DET1 lines.

Table 4-3 Ketocarotenoids, carotenoids and chlorophylls contents in CrtZW as control line and 11 CrtZW.DET1 lines at MG stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. Each sample had three technical replicates. CrtZW samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. Significant differences with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used.

| | **MG** | | **CrtZW.DET1** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **CrtZW** | **Line1** | **Line 2** | **Line 3** | **Line 4** | **Line 5** | **Line6** | **Line 7** | **Line 8** | **Line 9** | **Line 10** | **Line 11** |
| | **Chlorophyll a** | 12.2±8.59 | 11.58±1.12 | **32.15±0.24 ***** | **37.43±4.3 ***** | **34.29±3.04 ***** | 13.29±1.78 | - | 19.9±1.11 | 6.86±0.26 | 12.33±0.87 | - | 12.04±1.66 |
| | **Lutein** | 6.02±0.12 | - | **10±0.17 ***** | **7.8±0.22 **** | **11.37±0.62 ***** | **10.2±1.39 ***** | **7.09±0.31 *** | **8.14±1.16 **** | **8.41±0.21 **** | **9.3±0.32 ***** | **7.33±0.05 *** | **9.27±0.35 ***** |
| | **Chlorophyll b** | 100.58±17.5 | **245.71±11.81 ***** | **485.87±24.41 ***** | **241.96±17.54 ***** | **454.17±13.75 ***** | **355.5±33.51 ***** | 19.58±2.64 | **367.39±10.3 ***** | **293.85±11.55 ***** | **382.29±12.7 ***** | 2.72±1 | **388.46±14.75 ***** |
| **Ketocarotenoids** | **Astaxanthin** | 16.12±6.57 | 33.93±3.48 | 17.16±1.2 | 26.59±2.44 | 30.76±1.16 | 31.89±3.03 | 18.48±0.86 | 16.18±2.73 | 18.01±1.65 | 18.41±1.52 | 27.33±0.44 | 17.61±1.55 |
| | **Adonixanthin** | 4.03±0.72 | 6.77±0.2 | 5.09±0.33 | 7.25±0.54 | 6.21±0.64 | 11.78±1.33 | 8.12±0.31 | 5.49±1.15 | 6.52±0.41 | 5.82±0.47 | 12.94±0.39 | 5.17±0.38 |
| | **Adonirubin** | 21.61±2.28 | **59.57±6.56 ***** | **73.64±1.33 ***** | **50.01±3.73 **** | **71.78±4.12 ***** | **64.47±4.98 ***** | **42.2±2.01 *** | **53.56±1.07 **** | **54.5±2.97 **** | **46.66±1.23 *** | **45.4±1.67 *** | **47.62±2.2 *** |
| | **Unknown 1** | 4.45±0.77 | 5.48±0.45 | **26.62±0.71 ***** | **11.64±2.27 *** | **21.52±0.52 ***** | **14.65±0.46 **** | 6.37±0.3 | **13.73±0.85 **** | **13.07±0.83 **** | **17.98±0.75 ***** | **7.79±0.16 *** | **17.61±0.27 ***** |
| | **Canthaxanthin** | 7.93±1.21 | 19.46±0.81 | **39.26±1.65 ***** | 20.18±1.5 | **27.09±0.75 **** | **26.89±3.65 **** | 17.65±1.23 | **25.06±1.08 **** | **27.19±1.61 **** | **26.06±1.67 **** | 17.38±0.38 | **24.18±0.51 *** |
| | **Echineone** | 3.09±0.21 | 5.54±0.17 | 7.57±0.18 | 4.87±0.34 | 4.82±0.37 | 4.88±0.52 | 4.28±0.05 | 4.86±0.44 | 5.38±0.14 | 5.34±0.21 | 4.25±0.07 | 5.08±0.06 |
| | **Unknown 2** | 2.16±0.22 | 3.16±0.68 | 1.69±0.03 | 6.67±1.38 | - | 2.3±0.04 | 2.77±0.06 | 1.64±0.15 | 1.81±0.03 | 1.79±0.02 | 3.2±0.05 | 2.26±0.02 |
| | **Unknown 3** | 2.81±0.38 | 7.25±0.36 | 2.72±0.11 | 3.11±0.17 | 2.77±0.09 | 3.43±0.16 | 7.47±0.29 | 2.61±0.23 | 3.75±0.11 | 2.77±0.12 | 6.89±0.32 | 2.41±0.02 |

Table 4-4 Ketocarotenoids, carotenoids and chlorophylls contents in CrtZW as control line and 11 CrtZW.DET1 lines at 3 Phen stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. Each sample had three technical replicates. CrtZW samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used.

| | **3 Phen** | | **CrtZW.DET1** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **CrtZW** | **Line1** | **Line 2** | **Line 3** | **Line 4** | **Line 5** | **Line6** | **Line 7** | **Line 8** | **Line 9** | **Line 10** | **Line 11** |
| | **Chlorophyll a** | 11.73±6.76 | - | 17.19±0.16 | 4.61±0.34 | **21.2±1.31 **** | - | - | - | - | - | - | - |
| | **Lutein** | 5.72±0.18 | **-** | **9.03±0.27 **** | **8.63±0.45 **** | **12.99±0.04 ***** | 7.24±0.17 | 6.18±0.05 | 5.63±0.02 | 6.44±0.06 | 6.55±0.09 | 6.22±0.07 | 7.37±0.1 |
| | **Chlorophyll b** | 52.96±11.96 | 73.03±16.28 | **216.89±7.34 ***** | **202.91±19.04 ***** | **343.85±13.37 ***** | **9±2.42 ***** | - | - | - | - | - | - |
| **Ketocarotenoids** | **Astaxanthin** | 19.5±4.21 | **31.24±0.71 ***** | **35.33±1.35 ***** | **39.69±3.29 ***** | **53.94±0.56 ***** | 25.53±0.52 | 24.08±1.88 | 26±1.65 | 19.1±0.88 | 24.78±0.62 | **34.77±0.96 ***** | **29.96±2 ***** |
| | **Adonixanthin** | 4.35±0.97 | **7.72±0.39 ***** | **8.47±0.27 ***** | **10.2±0.97 ***** | **28.83±1.57 ***** | **10.36±0.33 ***** | **10.82±0.66 ***** | **6.48±0.74 *** | **10.15±0.29 ***** | **10.45±0.06 ***** | **14.61±0.22 ***** | **12.69±0.39 ***** |
| | **Adonirubin (Phenicoxanthin)** | 19.81±2.1 | **48.28±1.66 ***** | **55.1±2.04 ***** | **67.27±6.31 ***** | **103.19±3.77 ***** | **46.25±1.46 ***** | **46.34±3.09 ***** | **36.56±1.91 ***** | **47.15±0.86 ***** | **30.8±0.11 ***** | **49.05±1.83 ***** | **42.37±2.29 ***** |
| | **Unknown1** | 2.53±0.44 | **-** | 9.86±0.39 | 6.82±0.33 | **20.74±1.11 ***** | 8.42±0.49 | 4.58±0.21 | 4.96±0.25 | 8.46±0.15 | 7.28±0.26 | 4.87±0.37 | 9.63±0.55 |
| | **Canthaxanthin** | 6.71±1.03 | **17.39±0.51 ***** | **22.83±1.02 ***** | **21.54±1.9 ***** | **20.09±0.28 ***** | **17.29±0.24 ***** | **17.79±1 ***** | **12.92±0.54 ***** | **22.37±0.86 ***** | **11.91±0.34 ***** | **17.62±0.79 ***** | **14.92±0.62 ***** |
| | **Echineone** | 2.92±0.23 | 5.05±0.18 | 5.27±0.16 | 4.94±0.34 | 4.12±0.26 | 4.19±0.05 | 4.32±0.11 | 3.61±0.13 | 4.78±0.13 | 3.42±0.02 | 4.36±0.15 | 3.86±0.06 |
| | **Unknown2** | 3.44±0.67 | **5.42±0.19 ***** | **4.49±0.09 *** | **5.74±0.44 ***** | **7.28±0.29 ***** | 2.87±0.09 | 4.4±0.22 | 3.14±0.15 | 3.71±0.02 | 3.45±0.1 | **6.3±0.12 ***** | 4.15±0.2 |
| | **Unknown3** | 6.41±3.18 | **13.34±0.45 ***** | 8.43±0.71 | 6.19±0.44 | **17.53±0.75 ***** | 7.7±1.22 | **12.78±0.53 ***** | 4.74±0.29 | **15.81±0.41 ***** | 5.44±0.22 | **11.51±0.44 *** | 5.29±0.28 |

Table 4-5 Ketocarotenoids and carotenoids contents in CrtZW as control line and 11 CrtZW.DET1 lines at 7 dpb stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. Each sample had three technical replicates. CrtZW samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "- " Indicates pigment was not detectable at the concentration used.

| | **Ripe** | | **CrtZW.DET1** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **CrtZW** | **Line1** | **Line 2** | **Line 3** | **Line 4** | **Line 5** | **Line6** | **Line 7** | **Line 8** | **Line 9** | **Line 10** | **Line 11** |
| | **Lycopene** | 1006.02±191.74 | 1135.22±28.58 | **2245.97±180.56** *** | 1039.75±33.6 | **1500.72±50.77** *** | **1734.75±61.82** *** | 948.29±23.32 | **1519.99±85.94** *** | **590.63±10.7** ** | 830.07±45.87 | 844.78±70.74 | 1241.12±55.91 |
| **Ketocarotenoids** | **Astaxanthin** | 38.24±16.38 | 75.01±22.88 | **145.57±16.08** *** | **95.6±2.17** ** | **102.4±14.49** ** | **86.07±5.11** * | 66.51±1.13 | 70.78±0.79 | 54.98±0.6 | 47.34±6.97 | 54.26±1.25 | 74.35±12.47 |
| | **Adonixanthin** | 6.03±2.45 | 8.84±4.24 | **23.28±11.86** * | **32.03±2.42** ** | **33.61±1.46** ** | 9.34±4.83 | 11.26±0 | 6.03±4.15 | 8.33±0.95 | 20.74±4.64 | 6.42±0.38 | 10.96±5.36 |
| | **Adonirubin** | 43.82±11.23 | **139.49±5.72** *** | **190.22±15.89** *** | **191.64±10.17** *** | **162.51±23.71** *** | **102.34±2.05** *** | **106.46±2.77** *** | **99.24±5.27** *** | **96.28±4.13** *** | **76.56±4.62** * | **79.46±0.56** ** | **130.88±12.29** *** |
| | **Unknown1** | - | - | - | **14.34±0.41** *** | **2.73±0.94** *** | - | - | - | - | - | - | - |
| | **Canthaxanthin** | 19.09±2.04 | **54.59±1.85** *** | **80.65±5.2** *** | **80.3±5.87** *** | **56.92±7.6** *** | **37.38±1.37** *** | **47.89±3.12** *** | **49.53±1.79** *** | **48.03±1.4** *** | 22.8±1.55 | **29.6±2.11** * | **34.44±5.76** *** |
| | **Echineone** | - | - | - | **11.3±1.16** ** | **6.82±0.9** * | 7.64±0.7 | 8.97±0.38 | **5.75±0.48** * | **6.34±0.32** * | **7.06±1.22** * | 4.56±0 | 7.42±0.93 |
| | **Unknown2** | - | **11.74±0.53** *** | - | - | **3.82±0.33** *** | **9.13±0.75** *** | **7.11±0.08** *** | **5.6±0.61** *** | **5.93±0.66** *** | **11.8±1.23** *** | **7.42±0.32** *** | **9.3±0.65** *** |
| | **Unknown3** | 6.81±0.78 | 17.58±1 | 11.18±4.09 | 13.27±1.26 | 17.77±2.5 | 15.52±1.17 | 12.76±1.07 | 8.71±1.36 | 15.77±1.41 | **20.13±2.71** * | 11.59±0.75 | 12.1±2.45 |

Table 4-6 Ketocarotenoids, carotenoids and chlorophylls contents in 2 selected CrtZW.DET1 lines and its parental and wild type control lines at MG stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. Each sample had three technical replicates. Other samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used.

| | MG | T56 | Money maker (MM) | DET1 | CrtZW | CrtZW.DET1 line2 | CrtZW.DET1 line4 |
|---|---|---|---|---|---|---|---|
| | **Chlorophyll a** | 25.73±1.43 | 21.4±10.14 | 35.97±17.77 | 12.2±8.59 | 32.15±0.24 | 34.29±3.04 |
| | **Lutein** | 19.17±10.43 | 15.93±1.4 | 38.21±9.79 | 6.02±0.12 | 10±0.17 | 11.37±0.62 |
| | **Chlorophyll b** | 128.52±4.49 | 120.86±45.62 | 496.03±72.06 | 100.58±17.5 | **485.87±24.41 ***** | **454.17±13.75 **** |
| | **β-carotene** | 9.75±1.93 | 9.21±1.1 | 22.86±4.04 | - | - | - |
| **Ketocarotenoids** | **Astaxanthin** | - | - | - | 16.12±6.57 | **17.16±1.2** | **30.76±1.16 ***** |
| | **Adonixanthin** | - | - | - | 4.03±0.72 | **5.09±0.33 **** | **6.21±0.64 ***** |
| | **Adonirubin** | - | - | - | 21.61±2.28 | **73.64±1.33 ***** | **71.78±4.12 ***** |
| | **Unknown 1** | - | - | - | 4.45±0.77 | **26.62±0.71 ***** | **21.52±0.52 ***** |
| | **Canthaxanthin** | - | - | - | 7.93±1.21 | **39.26±1.65 ***** | **27.09±0.75 ***** |
| | **Echinoene** | - | - | - | 3.09±0.21 | **7.57±0.18 ***** | **4.82±0.37 ***** |
| | **Unkown 2** | - | - | - | 2.16±0.22 | **1.69±0.03 ***** | - |
| | **Unkown 3** | - | - | - | 2.81±0.38 | 2.72±0.11 | 2.77±0.09 |

Table 4-7 Ketocarotenoids and carotenoids contents in 2 selected CrtZW.DET1 lines and its parental and wild type control lines at ripe stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. Each sample had three technical replicates. Other samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. Significant differences with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used.

| | Ripe | T56 | Money maker (MM) | DET1 | CrtZW | CrtZW.DET1 line2 | CrtZW.DET1 line4 |
|---|---|---|---|---|---|---|---|
| | **Lutein** | 15.24±6.11 | 20.23±1.62 | 65.17±38.75 | - | - | - |
| | **Lycopene** | 513.3±253.69 | 871.81±229.01 | 1357.42±211.32 | 1006.02±191.74 | **2245.97±180.56 ***** | **1500.72±50.77 **** |
| | **β-carotene** | 39.98±12.21 | 46.98±13.54 | 185.21±51.84 | - | **13.89±0.78 ***** | - |
| **Ketocarotenoids** | **Astaxanthin** | - | - | - | 38.24±16.38 | **145.57±16.08 ***** | **102.4±14.49 ***** |
| | **Adonixanthin** | - | - | - | 6.03±2.45 | **23.28±11.86 **** | **33.61±1.46 ***** |
| | **Adonirubin** | - | - | - | 43.82±11.23 | **190.22±15.89 ***** | **162.51±23.71 ***** |
| | **Unknown 1** | - | - | - | - | - | **2.73±0.94 ***** |
| | **Canthaxanthin** | - | - | - | 19.09±2.04 | **80.65±5.2 ***** | **56.92±7.6 ***** |
| | **Echinoene** | - | - | - | - | - | **6.82±0.9 ***** |
| | **Unkown 2** | - | - | - | - | - | - |
| | **Unkown 3** | - | - | - | 6.81±0.78 | 11.18±4.09 | **17.77±2.5 ***** |

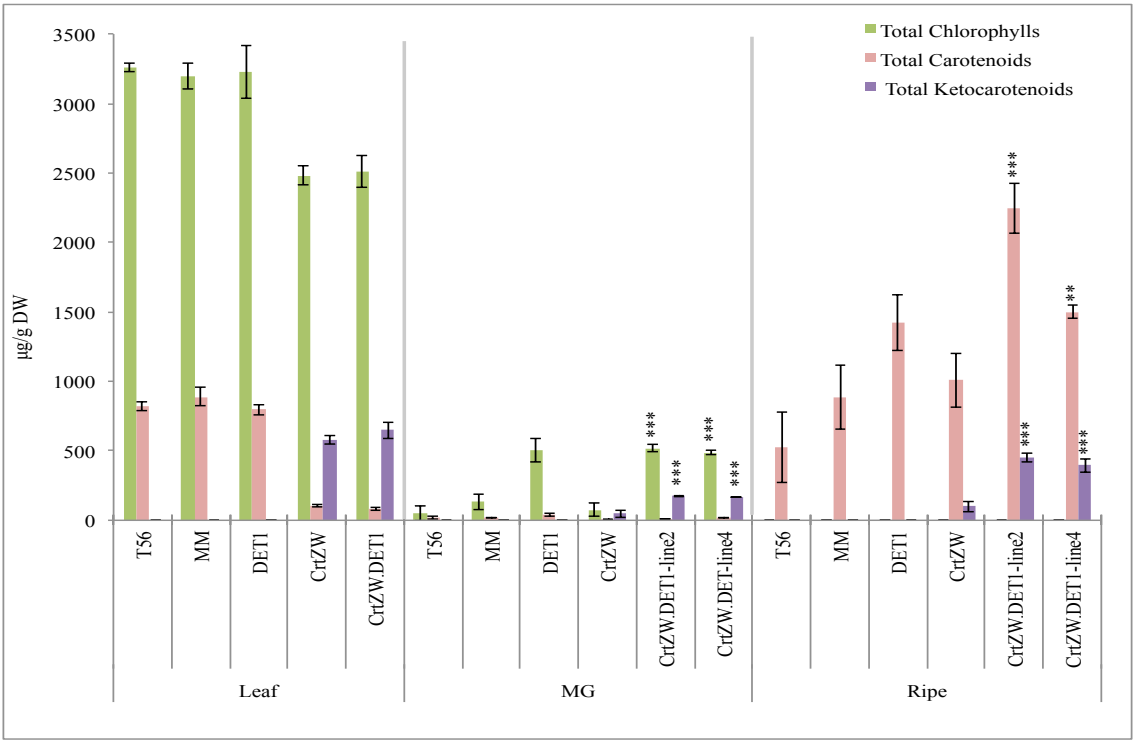



Figure 4-12 Total Ketocarotenoids, carotenoids and chlorophylls contents of F1 CrtZW.DET1 selected lines and its parental and wild type backgrounds lines at leaf, MG and Ripe stages.

Total Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. T56 and Money Maker (MM) were the wild type backgrounds; DET1 (T56) and CrtZW (MM) were the parental lines of CrtZW.DET1. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. Each sample had three technical replicates. Other samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). Error bars indicate ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. The significant differences just are shown for CrtZW.DET1 lines for clarity. Significant differences with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

#### 4.2.3.3 The selection of optimal lines from first generation pigment profile for CrtZW.APRR2 and its parental and background controls lines

The best lines were selected from 4 plants of the first generation CrtZW.APRR2 from their pigment profile. For pigment profile the UPLC-PDA analysis was performed as described in section 2.2.3. Pigment profile of leaf, MG and 7 dpb fruits were carried out for 4 CrtZW.APRR2 lines, the parental lines (CrtZW.Mic & APRR2) and the backgrounds variety of MicrotomMicrotom line. The ketocarotenoids and other pigment content for 4 CrtZW.APRR2 lines to its CrtZW comparator for MG and ripe stages are shown Table 4-8 and Table 4-9 respectively. For an overall view about the changes in CrtZW.APRR2 to its parental and wild type backgrounds, the pigment changes of the best-selected line of CrtZW.APRR2 and its parental and backgrounds lines at MG and ripe stage is illustrated in Figure 4-13. For selecting the best line, the ketocarotenoids content changes at all stages of ripening, changes in chlorophylls levels at MG stage and lycopene content at ripe stage were considered for best line selection. In overall the CrtZW.APRR2 lines does not show a considerable improvement in ketocarotenoids, chlorophylls and lycopene level to CrtZW parental line. The line 3 showed a slight change in all of ketocarotenoids level at MG stage but the chlorophylls level did not change to CrtZW (Table 4-8). At ripe stage the lycopene level decreased in some lines but line 3 indicated the highest level of lycopene among other lines, which was close to its CrtZW line. A small increase of 0.5 fold in astaxanthin and adonirubin were observed at this stage and adonixanthin doubled in comparison to CrtZW (see Table 4-9). Although a significant change was not detected in the new phenotypes of CrtZW.APRR2, but line 3 presented the same amount of endogenous pigments and a slight increase of ketocarotenoids to CrtZW. Therefore, the line 3 of CrtZW. APRR2 were chosen for subsequent steps of experiments in F2 generation based on the best increased level of pigments in different stages of ripening (see the overall view in Figure 4-13). The seeds of this line sowed for growing the best F2 plants of CrtZW.APRR2 lines. The most important data are shown in Figure 4-13 for a better overall concept.

Table 4-8 Ketocarotenoids, carotenoids and chlorophylls contents in CrtZW as control line and 4 CrtZW.APRR2 lines at MG stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.APRR2 samples were made from pool of three fruits for each line. Each sample had three technical replicates. Other samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.APRR2 lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used.

| | **MG** | | | | **CrtZW.APRR2 Lines** | | | |
|---|---|---|---|---|---|---|---|---|
| | | **APRR2 (MicrotomMicrotom)** | **APRR2** | **CrtZW.Mic** | **Line1** | **Line2** | **Line3** | **Line4** |
| | **Chlorophyll a** | 19.06±2.32 | 31.52±11.95 | 12.53±4.98 | 15.97±0.13 | - | - | - |
| | **Lutein** | 28.13±2.63 | 46.95±6.03 | 5.65±0.23 | 6.92±0.16 | 5.36±0.03 | 6.46±0.13 | 5.54±0.05 |
| | **Chlorophyll b** | 215.13±38.37 | 476.55±59.52 | 45.75±11.21 | **147.07±3.16** ** | 21.07±0.01 | 30.8±4.78 | 21.18±1.62 |
| | **β-carotene** | 20.55±2.41 | 25.16±1.9 | - | - | - | - | - |
| **Ketocarotenoids** | **Astaxanthin** | - | - | 8.95±2.31 | **15.47±0.06** *** | 11.12±0.54 | **18.52±0.74** *** | **12.97±0.78** *** |
| | **Adonixanthin** | - | - | 3.88±0.31 | **8.59±0.03** *** | 4.2±0.14 | **5.78±0.14** *** | **6.78±0.4** *** |
| | **Adonirubin** | - | - | 13.78±2.08 | **34.62±1.18** *** | 12.39±0.3 | **24.19±0.69** *** | **13.67±1.35** |
| | **Unknown 1** | - | - | 1.93±0.11 | **4.62±0.34** *** | **1.76±0.06** ** | **2.89±0.13** *** | **1.82±0.11** |
| | **Canthaxanthin** | - | - | 6.5±0.46 | **15.37±0.28** *** | **4.44±0.08** *** | **9.2±0.26** *** | **5.01±0.32** *** |
| | **Echineone** | - | - | 2.89±0.1 | **4.13±0.09** *** | **2.57±0.08** *** | **3.19±0.1** *** | **2.67±0.09** ** |
| | **Unknown 2** | - | - | 1.76±0.14 | 1.9±0.06 | 1.92±0.01 | **2.1±0.01** *** | **2.05±0.09** *** |
| | **Unknown 3** | - | - | 2.4±0.08 | **3.03±0.08** *** | **3.1±0.09** *** | **3.35±0.06** *** | **3.98±0.24** *** |

Table 4-9 Ketocarotenoids and carotenoids contents in CrtZW as control line and 4 CrtZW.APRR2 lines at Ripe stage.

Ketocarotenoids and Carotenoids contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.APRR2 samples were made from pool of three fruits for each line. Each sample had three technical replicates. Other samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.APRR2 lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used.

| | **Ripe** | | | | **CrtZW.APRR2 Lines** | | | |
|---|---|---|---|---|---|---|---|---|
| | | **APRR2 (MicrotomMicrotom)** | **APRR2** | **CrtZW.Mic** | **Line1** | **Line2** | **Line3** | **Line4** |
| | **Lutein** | 19.88±2.27 | 22.92±1.92 | - | - | - | - | - |
| | **Lycopene** | 1008.23±106.16 | 1047.63±90.55 | 920.94±56.54 | **516.99±35.16** *** | 956.37±95.73 | 1093.43±33.98 | 781.86±2.73 |
| | **β-carotene** | 50.3±12.91 | 45.42±6.15 | - | 7.05±0.2 | 6.72±0.07 | 8.49±0.19 | 7.1±0.44 |
| **Ketocarotenoids** | **Astaxanthin** | - | - | 29.64±2.15 | **44.66±2.84** *** | **24.33±2.06** *** | **40.89±1.26** *** | **21.03±0.9** *** |
| | **Adonixanthin** | - | - | 4.23±1.07 | **13.01±0.89** *** | 5.29±0.93 | **8.7±1.14** *** | **6.39±0.15** ** |
| | **Adonirubin** | - | - | 21.22±2.55 | **36.71±2.02** *** | **16.46±2.39** ** | **26.82±1.01** *** | **12.7±0.69** *** |
| | **Canthaxanthin** | - | - | 10.02±0.62 | 10.44±0.92 | 7.53±0.8 | 11.68±1 | **4.02±0.23** ** |
| | **Unknown 2** | - | - | 5.63±0.97 | 6.71±0.18 | 5.53±0.3 | 6.31±0.22 | **6.77±0.28** * |
| | **Unknown 3** | - | - | 2.82±0.35 | **4.05±0.23** *** | 2.63±0.12 | **2.36±0.09** * | 2.95±0.1 |

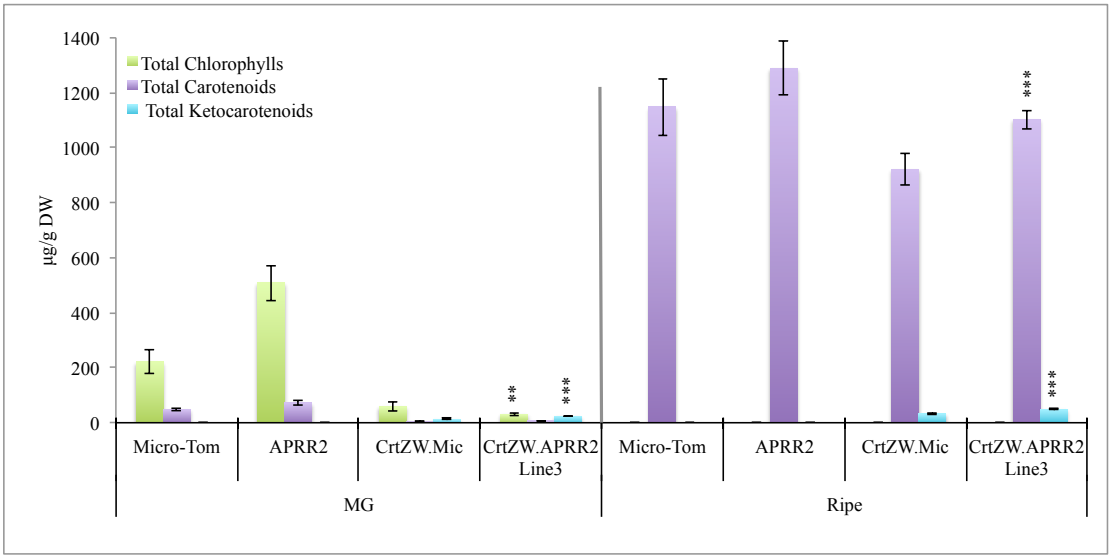



Figure 4-13 Total Ketocarotenoids, carotenoids and chlorophylls contents of F1 CrtZW.APRR2 selected line and its parental and wild type backgrounds lines at MG and Ripe stages.

Total Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Micro-Tom was the wild type background; APRR2 and CrtZW.Mic were the parental lines of CrtZW.APRR2. Methods used for determinations are described in section 2.2.3. Each CrtZW.APRR2 sample was made from pool of three fruits for each line. Each sample had three technical replicates. Other samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). Error bars indicate ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW.Mic and CrtZW.APRR2 lines. The significant differences just are shown for CrtZW.APRR2 lines for clarity. Significant differences with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

### 4.2.4 Second generation pigment profile

#### 4.2.4.1 Analysis of carotenoid and ketocarotenoids pigments in the CrtZWDET1 line and its derived control lines of F2 generation

Pigment analysis for F2 offspring were performed on tomato fruit from the selected CrtZW.DET1 lines and its derived comparators. Fruit samples were collected throughout fruit developmental stages of MG, 3 dpb and Ripe steps. A pool of three fruits for each of three biological replicate was prepared and the samples were extracted as described in section 2.2.1.1. Pigment extracts were separated and quantified by UPLC-PDA. Quantitative and qualitative changes in carotenoid content were observed throughout the developmental and ripening stages analysed. The detailed changes of pigments in CrtZW.DET1 (T56.MM) line and its derived parental lines of DET1 (T56.MM) and CrtZW (T56.MM) lines and the derived wild type

background Control (T56.MM) is illustrated in Table 4-10, Table 4-11 and Table 4-12 at MG, 3 dpb and ripe stage respectively for a comprehensive comparison.

In CrtZW.DET1 line at MG stage the first impression was the overall increase of all of pigments. The chlorophyll a and b increased by 14.5 and 6.3 fold to CrtZW respectively. α-Tocopherol as one of the precursor of carotenoid pathway, similar to its parental DET1 line, in CrtZW.DET1 line increased 2.6 times to CrtZW. Ketocarotenoids level grew from 1.5 fold (unknown 2) to 3.7 fold (canthaxanthin) in CrtZW.DET1 phenotype to CrtZW and no β-carotene was detected in neither of these two lines (Table 4-10). On the whole, the considerable increase of 6.9 fold, 2 fold and 2.6 fold in total chlorophylls, total carotenoids and total ketocarotenoids is a considerable improvement for the new variety of CrtZW.DET1 at MG stage (Table 4-10).

In CrtZW.DET1 line at 3 dpb stage no difference observed in lycopene level and also in total carotenoids level although total ketocarotenoids showed a 2.6 times increase to CrtZW. As this stage is a passing stage, which many changes occur at this step, it is better to focus on MG and Ripe stage for concluding about the real changes in this new line (Table 4-11).

At the ripe stage important increases were observed in many of pigments in CrtZW.DET1. α-Tocopherol, phytoene and phytofluene increased 4, 1.2 and 2.4 fold to CrtZW comparator respectively. An overall increase of ketocarotenoids was observed as well, for instance astaxanthin, adonirubin and canthaxanthin elevated 3, 3.3 and 2.5 times to CrtZW line. This active production of ketocarotenoids leads to accumulation of very small amounts of lutein and β-carotene. This small amount of lutein and β-carotene pigments is an achievement for CrtZW.DET1 line in comparison to zero content of CrtZW line. This production of lutein and β-carotene is significant in due of its DET1 parental line. The most important indigenous carotenoid in tomato fruits, lycopene, increased up to 1.5 fold at ripe stage. This improvement in both total carotenoids (1.5 fold increase) and total ketocarotenoids (2.7 fold increase) is a valuable result for this new variety (Table 4-12). The most important data are shown in Figure 4-14 for a better visual concept. For discussion of results of this section alongside with other experiments see sections 4.7.1.1, 4.7.1.4 and 4.7.1.6.

Table 4-10 Ketocarotenoids, carotenoids and chlorophylls contents in F2 CrtZW.DET1 line and its derived parental and control lines at MG stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each sample was made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used. The black stars indicate significant differences to Control (T56.MM) and the blue stars indicate a significant difference compared to CrtZW line.

| | MG | Control (T56.MM) | DET1 (T56.MM) | CrtZW (T56.MM) | CrtZW.DET1 (T56.MM) |
|---|---|---|---|---|---|
| | **Chlorophyll a** | - | **25.67±3.04** *** | 4.8±0.16 | **70.03±4.01** *** |
| | **Lutein** | 20.13±0.16 | **82.1±3.2** *** | **7.82±0.38** *** | **16.31±1.2**** |
| | **Chlorophyll b** | 43.24±8.12 | **565.6±29.66** *** | 64.06±0.68 | **405.63±15.47** *** |
| | **α-tocopherol** | 77.14±0.94 | **171.69±8.2** *** | 71.74±1.3 | **189.92±6.94** *** |
| | **Pheophytin b** | 46.35±1.4 | **23.93±0.35** *** | **7.2±0.33** *** | **11.26±0.95** ** |
| | **β-carotene** | 12.9±0.64 | **49.04±2.69** *** | - | - |
| **Ketocarotenoids** | **Astaxanthin** | - | - | 11.42±0.17 | **26.18±1.07** *** |
| | **Adonixanthin** | - | - | 6.99±0.32 | **11.23±0.59** *** |
| | **Adonirubin** | - | - | 22.28±0.42 | **55.26±2.67** *** |
| | **Unknown 1** | - | - | 4.11±0.44 | **17.26±0.59** *** |
| | **Canthaxanthin** | - | - | 6.97±0.05 | **26.16±0.97** *** |
| | **Echineone** | - | - | 2.87±0.1 | **5.7±0.22** *** |
| | **Unknown 2** | - | - | 1.6±0.04 | **2.42±0.1** *** |
| | **Unknown 3** | - | - | 2.77±0.07 | **4.36±0.08** *** |
| | **Total Chlorophylls** | 43.24±8.12 | **591.27±31.6** *** | 68.85±0.55 | **475.67±18.47** *** |
| | **Total Carotenoids** | 33.03±0.63 | **131.14±5.89** *** | **7.82±0.38** *** | **16.31±1.2**** |
| | **Total Ketocarotenoids** | - | - | 58.05±0.91 | **148.56±5.93** *** |

Table 4-11 Ketocarotenoids, carotenoids and chlorophylls contents in F2 CrtZW.DET1 line and its derived parental and control lines at 3 dpb stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each sample was made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used. The black stars indicate significant differences to Control (T56.MM) and the blue stars indicate a significant difference compared to CrtZW line.

| | 3 dpb | Control (T56.MM) | DET1 (T56.MM) | CrtZW (T56.MM) | CrtZW.DET1 (T56.MM) |
|---|---|---|---|---|---|
| | **Lutein** | 21.92±0.86 | **65.66±2.28** *** | **6.73±0.4** *** | **11.41±0.77** * |
| | **Chlorophyll b** | - | - | - | **9.67±0.67** *** |
| | **Lycopene** | 118.5±8.52 | **420.2±16.46** *** | **65.53±4.21** ** | 59.79±3.92 |
| | **α-tocopherol** | 147.12±9.54 | **360.25±10.38** ** | 148.98±10.73 | **507.95±29.71** *** |
| | **Pheophytin b** | 18.99±0.59 | **37.51±1.54** *** | **35.92±1.91** *** | **79.21±3.77** *** |
| | **β-carotene** | 38.28±2.49 | **99.56±4.2** *** | **8.75±0.26** *** | 8.51±0.57 |
| **Ketocarotenoids** | **Astaxanthin** | - | - | 19±1.25 | **48.19±3.04** *** |
| | **Adonixanthin** | - | - | 6.49±0.28 | **18±1.12** *** |
| | **Adonirubin** | - | - | 29.28±1.64 | **90.67±8** *** |
| | **Unknown 1** | - | - | 2.81±0.47 | **9.92±0.77** *** |
| | **Canthaxanthin** | - | - | 15.11±0.71 | **40.86±2.59** *** |
| | **Echineone** | - | - | 4.89±0.24 | **7.61±0.47** *** |
| | **Unknown 2** | - | - | 5.14±0.12 | **6.58±0.39** *** |
| | **Unknown 3** | - | - | 9.92±0.32 | **15.22±1.82** ** |
| | **Total Chlorophylls** | - | - | - | **9.67±0.67** *** |
| | **Total Carotenoids** | 178.69±11.69 | **585.42±19.82** *** | **78.09±7.05** *** | 79.71±5.18 |
| | **Total Ketocarotenoids** | - | - | 91.68±5.01 | **237.04±17.92** *** |

Table 4-12 Ketocarotenoids, carotenoids and chlorophylls contents in F2 CrtZW.DET1 line and its derived parental and control lines at ripe stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each sample was made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used. The black stars indicate significant differences to Control (T56.MM) and the blue stars indicate a significant difference compared to CrtZW line.

| | Ripe | Control (T56.MM) | DET1 (T56.MM) | CrtZW (T56.MM) | CrtZW.DET1 (T56.MM) |
|---|---|---|---|---|---|
| | **Phytoene** | 5.27±0.21 | **7.15±0.81** * | **10.28±0.45** *** | **12.7±0.17** ** |
| | **Phytofluene** | 73.75±4.62 | **146.27±7.38** *** | **115.41±1.32** *** | **281.51±4.81** *** |
| | **Lutein** | 25.5±1.48 | **79.09±1.81** *** | - | **8.45±0.32** * |
| | **Lycopene** | 688.01±71.41 | **1258.74±62.98** *** | **1185.92±25.19** *** | **1728.99±24.24** *** |
| | **α-tocopherol** | 217.69±20.56 | **491.81±28.91** *** | **148.27±1.61** * | **602.11±7.97** *** |
| | **β-carotene** | 55.31±6.69 | **188.18±9.83** *** | - | **29.34±1.7** * |
| **Ketocarotenoids** | **Astaxanthin** | - | - | 30.36±0.48 | **89.56±0.9** *** |
| | **Adonixanthin** | - | - | 8.76±0.55 | **25.29±0.88** *** |
| | **Adonirubin** | - | - | 28.86±1.8 | **96.35±1.58** *** |
| | **Unknown 1** | - | - | | 4.36±2.2 |
| | **Canthaxanthin** | - | - | 13.28±0.2 | **33.31±0.87** *** |
| | **Echineone** | - | - | 6.28±0.47 | - |
| | **Unknown 2** | - | - | 4.34±0.27 | **6.67±0.11** *** |
| | **Unknown 3** | - | - | 7.97±0.09 | **13.39±0.86** *** |
| | **Total Carotenoids** | 847.84±79.33 | **1679.43±81.01** *** | **1314.52±28.01** *** | **2058.18±25.5** *** |
| | **Total Ketocarotenoids** | - | - | **100.47±2.36** *** | **270.29±3.24** *** |

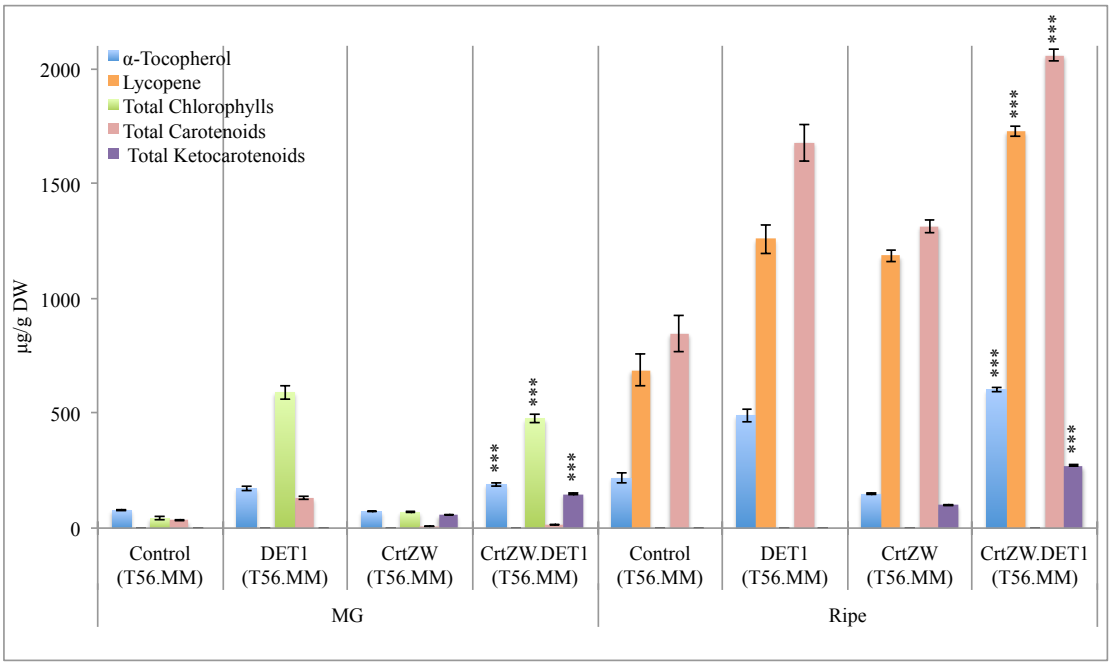



Figure 4-14 Total Ketocarotenoids, carotenoids and chlorophylls, α-tocopherol and lycopene contents of F2 CrtZW.DET1 line and its derived parental and control lines at MG and Ripe stages.

All of data are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.DET1 samples were made from pool of three fruits for each line. All of samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). Error bars indicate ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.DET1 lines. The significant differences just are shown for CrtZW.DET1 lines for clarity. Significant differences with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

#### 4.2.4.2 Analysis of carotenoid and ketocarotenoids pigments in the CrtZW.APRR2 lines and its control lines in the F2 generation

Pigment analysis for F2 generation were performed on tomato fruit from the selected line 3 of CrtZW.APRR2 and its derived comparators. Fruit samples were collected throughout fruit developmental stages of MG, 3 dpb and Ripe steps. A pool of three fruits for each of three independent biological replicate (see section 2.1.1) was created and the samples were extracted as described in section 2.2.1.1. Pigment extracts were separated and quantified by UPLC-PDA. Quantitative and qualitative changes in carotenoid content were observed throughout the developmental and ripening stages analysed. The detailed changes of pigments in CrtZW.APRR2 (Mic.MM) line and its derived parental lines of APRR2 (Mic.MM) and CrtZW (Mic.MM) lines and the derived wild type background Control (Mic.MM) is illustrated in Table 4-13, Table 4-14 and Table 4-15 at MG, 3 dpb and ripe stage respectively for a complete comparison.

In CrtZW.APRR2 at MG stage no significant changes observed in chlorophylls, carotenoid and ketocarotenoids content in CrtZW.APRR2 line to CrtZW comparator. In 3 dpb the most considerable changes in the overall increase of ketocarotenoids (1.6 fold).

In CrtZW.APRR2 at ripe stage α-tocopherol raised 1.9 fold to CrtZW comparator. No changes detected in phytoene, phytofluene, lutein and lycopene content. Small amount of β-carotene was detected in both CrtZW (Mic.MM) and CrtZW.APRR2 (Mic.MM) at ripe stage while did not detected in F1 CrtZW.Mic plants, but this amount is well bellow the wild type control content (6 fold lower) which is in due of derived pathway of ketocarotenoids biosynthesis from β-carotene. An overall modest increase in ketocarotenoids content were observed at this stage for instance adonirubin and astaxanthin increased by 2 and 1.7 times to CrtZW. All in all total ketocarotenoids level increased 1.8 fold while no change was detected in total carotenoid level. These data represent the slight changes of new phenotype of CrtZW.APRR2, which is expected for this stage, as the parental line of APRR2 also did not show a considerable improvement in total carotenoids level. The most important data are shown in Figure 4-15 for a better visual concept. For result discussion of this line see section 4.7.2.

Table 4-13 Ketocarotenoids, Carotenoids and Chlorophylls contents in F2 CrtZW.APRR2 line and its derived parental and control lines at MG stage.

Ketocarotenoids, carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each sample was made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used. The black stars indicate significant differences to Control (Mic.MM) and the blue stars indicate a significant difference compared to CrtZW (Mic.MM) line.

| | MG | Control (Mic.MM) | APRR2 (Mic.MM) | CrtZW (Mic.MM) | CrtZW.APRR2 (Mic.MM) |
|---|---|---|---|---|---|
| | **Chlorophyll a** | 96.38±13.48 | 110.36±10.25 | **37.7±11.11** ** | 14.57±3.05 |
| | **Lutein** | 61.48±2.83 | **122.34±0.86** *** | **11.15±1.09** *** | 17.08±7.65 |
| | **Chlorophyll b** | 452.99±145.34 | **1000.54±57.27** ** | 144.26±11.39 | 252.61±169.07 |
| | **α-tocopherol** | 53.05±10.62 | 150.34±22.77 | 88.06±22.28 | 106.82±71.13 |
| | **Pheophytin b** | 42.41±51.11 | 75.85±41.79 | 8.15±0.78 | 15.96±8.26 |
| | **β-carotene** | 34.59±0.49 | **72.01±1.23** *** | **14.78±1.93** *** | - |
| **Ketocarotenoids** | **Astaxanthin** | - | - | 12.02±0.99 | 14.49±8.66 |
| | **Adonixanthin** | - | - | 8.59±0.59 | 8.44±4.46 |
| | **Adonirubin** | - | - | 36.79±3.47 | 46.23±28.64 |
| | **Unknown 1** | - | - | 9.77±0.95 | 12.78±8.96 |
| | **Canthaxanthin** | - | - | 25.55±2.54 | 28.39±17.26 |
| | **Echineone** | - | - | 5.24±0.35 | 5.51±2.26 |
| | **Unknown 2** | - | - | 2.85±0.5 | - |
| | **Unknown 3** | - | - | 4.27±0.51 | 2.88±1.2 |
| | **Total Chlorophylls** | 549.37±150.32 | **1110.9±50.87** *** | 181.96±21.82 | 182.98±183.47 |
| | **Total Carotenoids** | 96.07±2.87 | **194.35±1.76** *** | **25.93±3.02** *** | 17.08±7.65 |
| | **Total Ketocarotenoids** | - | - | 104.12±8.48 | 119.66±72.36 |

Table 4-14 Ketocarotenoids, carotenoids and chlorophylls contents in F2 CrtZW.APRR2 line and its derived parental and control lines at 3 dpb stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each sample was made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used. The black stars indicate significant differences to Control (Mic.MM) and the blue stars indicate a significant difference compared to CrtZW (Mic.MM) line.

| | 3 dpb | Control (Mic.MM) | APRR2 (Mic.MM) | CrtZW (Mic.MM) | CrtZW.APRR2 (Mic.MM) |
|---|---|---|---|---|---|
| | **Lutein** | 60.9±7.04 | **97.63±2.63** *** | **7.33±0.46** *** | **21.34±0.65** * |
| | **Chlorophyll b** | 7.35±5.16 | - | - | 10.65±2.67 |
| | **Lycopene** | 303.46±52.32 | 345.9±3.04 | 255.13±7.75 | **143.37±3.84** * |
| | **α-tocopherol** | 282.75±43.43 | 463.89±17.12 | 300.03±13.3 | 457.61±5.84 |
| | **Pheophytin b** | - | - | **56.93±3.5** *** | **158.08±12.06** *** |
| | **β-carotene** | 75.74±10.5 | 138.31±5.04 | - | 15.65±0.41 |
| **Ketocarotenoids** | **Astaxanthin** | - | - | **35.48±1.62** *** | **59.34±2.78** *** |
| | **Adonixanthin** | - | - | **12.27±0.99** *** | **26.41±0.97** *** |
| | **Adonirubin** | - | - | **66.7±3.64** *** | **122.61±6.09** *** |
| | **Unknown 1** | - | - | **11.87±0.32** *** | **5.26±0.64** *** |
| | **Canthaxanthin** | - | - | **49.22±2.72** *** | **74.11±2.79** *** |
| | **Echineone** | - | - | **8.98±0.68** *** | **11.93±0.48** *** |
| | **Unknown 2** | - | - | **5.22±0.08** *** | 5.2±0.85 |
| | **Unknown 3** | - | - | **18.59±2.64** *** | 22.44±2.07 |
| | **Total Chlorophylls** | 7.35±5.16 | | | 10.65±2.67 |
| | **Total Carotenoids** | 440.1±69.07 | 535.74±64.48 | **262.46±8.15** * | 180.35±4.77 |
| | **Total Ketocarotenoids** | | | **208.35±12.47** *** | **327.31±14.69** *** |

Table 4-15 Ketocarotenoids and carotenoids contents in F2 CrtZW.APRR2 line and it's derived parental and control lines at MG stage.

Ketocarotenoids, Carotenoids and chlorophylls contents are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each sample was made from independent pools of minimum three fruits from three plants (minimum 9 fruits). The mean data are shown with ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the lines. Significant differences with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively and in bold. "-" Indicates pigment was not detectable at the concentration used. The black stars indicate significant differences to Control (Mic.MM) and the blue stars indicate a significant difference compared to CrtZW (Mic.MM) line.

| | Ripe | Control (Mic.MM) | APRR2 (Mic.MM) | CrtZW (Mic.MM) | CrtZW.APRR2 (Mic.MM) |
|---|---|---|---|---|---|
| | **Phytoene** | 16.45±4.03 | 12.89±1.22 | 27.04±5.47 | 38.38±5.68 |
| | **Phytofluene** | 206.71±64.39 | 170.57±19.17 | 244.32±8.65 | 296.21±10.42 |
| | **Lutein** | 38.06±9.34 | **73.13±7.37** ** | **5.34±0.13** ** | 6.26±0.92 |
| | **Lycopene** | 1726.7±528.99 | 1631.72±144.96 | 2073.27±13.57 | 1942.51±127.48 |
| | **α-tocopherol** | 375.99±120.9 | **604.57±59.7** * | 288.35±12.14 | **539.89±11.83*** |
| | **β-carotene** | 88.53±32.65 | **153.78±22.06** * | **12.79±0.07** * | 15.61±0.81 |
| **Ketocarotenoids** | **Astaxanthin** | - | - | **59.19±2.18** *** | **99.09±3.97** *** |
| | **Adonixanthin** | - | - | **15.44±1.07** *** | **31.41±2.69** *** |
| | **Adonirubin** | - | - | **64.08±6.24** *** | **132.64±8.65** *** |
| | **Unknown 1** | - | - | 1.86±0.38 | **7.46±2.86** ** |
| | **Canthaxanthin** | - | - | **34.82±2.85** *** | **48.69±1.46** *** |
| | **Unknown 2** | - | - | **6.53±0.41** *** | 7.72±0.4 |
| | **Unknown 3** | - | - | 11.62±0.51 | 11.87±6.38 |
| | **Total Carotenoids** | 2076.46±638.07 | 2042.08±188.6 | 2360.98±13.21 | 2298.98±113.05 |
| | **Total Ketocarotenoids** | - | - | **189.05±14.91** *** | **338.88±25.36** *** |

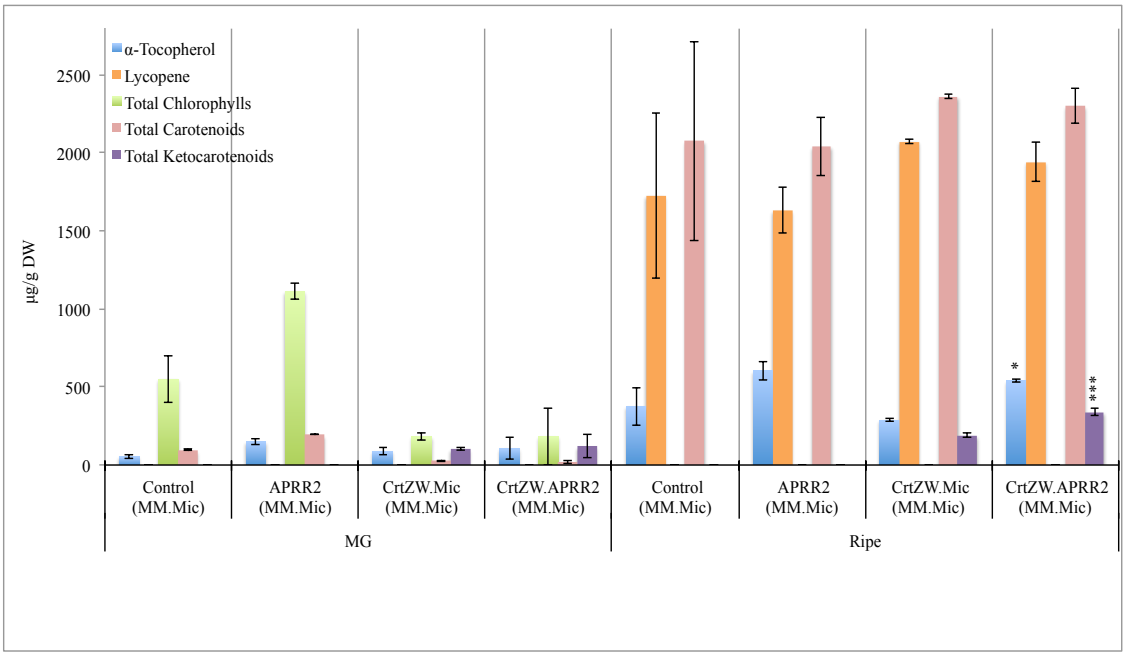



Figure 4-15 Total Ketocarotenoids, carotenoids and chlorophylls, α-tocopherol and Lycopene contents of F2 CrtZW.APRR2 line and its derived parental and control lines at MG and Ripe stages.

All of data are illustrated as µg/g DW. Methods used for determinations are described in section 2.2.3. Each CrtZW.APRR2 samples were made from pool of three fruits for each line. All of samples were made from independent pools of minimum three fruits from three plants (minimum 9 fruits). Error bars indicate ± SD. Anova test joined with Tukey post hoc test was used to determine significant differences between the parental CrtZW and CrtZW.APRR2 lines. The significant differences just are shown for CrtZW.APRR2 lines for clarity. Significant differences with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

### 4.2.5 Metabolite profile changes during fruit development and ripening in CrtZW.DET1 and derived comparator lines.

Changes in carotenoids and ketocarotenoid contents in the genotypes analysed and have been described in section 4.2.4.1. In addition to carotenoid changes, perturbations across intermediary metabolism were analysed by GC-MS among the $F_2$ generation of the ZW.DET1 line and its derived $F_2$ comparator lines (Control (T56.MM), CrtZW (T56.MM) and DET1 (T56.MM)). This experiment was performed for evaluating the other metabolite changes in the Q1968 line in addition to pigments. In section 3.2.3.4, it is shown there are some important genes with altered pattern of expression in the transcriptome which not only could affects on pigments but also other metabolites were detected. In section 3.3.3 based on transcriptome data, and alteration in metabolite level and pigments a conclusion made over the responsible genes. The metabolomics data generated was subjected to statistical

analyses and visualisation approaches. Over 50 metabolites were identified and quantified in 3 stages of fruit development and ripening in CrtZW.DET1 genotype and were compared to the control metabolites. As in this chapter the effect of *DET1* gene on the improvement of ketocarotenoid and carotenoid content and also their effect on the other metabolites was the predominant objective. To ensure accurate comparisons the CrtZW (T56.MM) line was considered the closest control. CrtZW (T56.MM) plants have the same backgrounds as the CrtZW.DET1 line and also contain the *CrtZW* genes, but not the *DET1* gene. Thus they are a suitable control for deducing the effect of the *DET1* gene. In addition for reference informative comparisons of CrtZW.DET1 with the background line of Control (T56.MM) and parental line of DET1 (T56.MM) are also explained in section 4.2.1.1. The analysed samples at 3dpb and ripe in CrtZW and CrtZW.DET1 lines were 3 Phen (18 dpb which phenotype is similar to wild type 3dpb) and 7 Phen (28 dpb which phenotype is similar to wild type 3dpb) as defined in section 4.2.1.3.

Most of changes in metabolites present in the CrtZW.DET1 in comparison to CrtZW line were observed firstly at the 3 dpb then ripe and MG stages respectively. As 3dpb is an intermediate stage, these vast changes are expected but because of variability of data at these stage for having a conclusion about metabolite alterations more focus on MG and ripe stages were done (see section 4.7.1.8). Amino acids were the most altered group of metabolites. Most of the observed alterations were due to increases in metabolites. Comparing CrtZW.DET1 to CrtZW line, the level of 3, 8 and 8 amino acids were increased at MG, 3 dpb and ripe stages respectively. In the CrtZW.DET1 line the proline and phenylalanine amino acids exhibited the highest rates of 8 and 7 fold increases respectively at 3 dpb; this highest rate at ripe stage belonged to glutamine and alanine with 10 and 8.4 fold growth respectively compared to CrtZW line. Serine, glutamine and alanine were elevated in all three stages of ripening (see Table 4-16). Organic acids were the second most altered groups of compounds. This group, like amino acids, were represented predominantly by increases. Some important organic acids in CrtZW.DET1 belonging to TCA cycle showed alterations. For instant citric acid increased in all of stages and aconitic acid accumulated 3.6 fold more at MG stage, on the contrary fumaric acid decreased in all of the stages in comparison to CrtZW line. Some other organic acids like aspartic acid and glucuronic acid also had an increase in all of stages of ripening. Considering the important role of glucuronic acid in plant cell wall, this increase is a significant finding. Other

metabolites also had important changes. Sucrose from sugar groups at MG stage increased by 4.6 fold. In general CrtZW.DET1 mostly make the differences in 3 dpb and ripe steps of ripening and the most varied class of compound was amino acids (see Table 4-16). For conclusion see section 4.7.1.8.

Table 4-16 The ratios of metabolite changes at different stages of fruit development and ripening in the F2 ZWDET1 line tomato fruits compared to its three derived comparators.

CONT: Control (T56.MM), ZW: CrtZW (T56.MM) and DET: DET1 (T56.MM). Data obtained from GC-MS analytical platform with subsequent statistical analysis. The data are presented as mean ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively. If a metabolite is unique to ZWDET1 at the concentration used is shown as In Inc (indefinite increase); and when is unique to a comparator line at the sample concentration used is shown as In Dec (indefinite decrease) and "-" indicates metabolite was not detectable in both ZWDET1 and the comparator at the sample concentration used. GABA: γ-Aminobutyric acid.

| Metabolite | Ratio | MG | 3 dpb | Ripe |
|---|---|---|---|---|
| **Amion Acid** | | | | |
| Alanine | ZWDET/CONT | **In Dec** | 2.46±0.65 | **4.69±1 *** |
| | ZWDET/ZW | - | **In Inc** | **8.42±1.8 *** |
| | ZWDET/DET | **In Dec** | **In Inc** | **6.11±1.3 *** |
| Asparagine | ZWDET/CONT | - | - | 0.74±0.12 |
| | ZWDET/ZW | - | **In Dec** | **In Inc** |
| | ZWDET/DET | **In Dec** | **In Dec** | 0.61±0.1 |
| Dihydrouracil | ZWDET/CONT | 0.13±0.02 | 1.14±0.5 | 0.69±0.08 |
| | ZWDET/ZW | **1.89±0.27 *** | 1.92±0.85 | 1.51±0.18 |
| | ZWDET/DET | **0.17±0.02 **** | 0.71±0.31 | 0.98±0.11 |
| Glutamine | ZWDET/CONT | **0.19±0.04 **** | 0.79±0.09 | 0.62±0.09 |
| | ZWDET/ZW | **In Inc** | **2.46±0.27 *** | **9.97±1.42 *** |
| | ZWDET/DET | **0.25±0.06 ***** | 0.62±0.07 | 1.15±0.16 |
| Glycine | ZWDET/CONT | 0.6±0.24 | **0.76±0.09 *** | 0.65±0.12 |
| | ZWDET/ZW | 1.57±0.63 | **3.73±0.42 **** | 1.46±0.27 |
| | ZWDET/DET | 0.86±0.34 | 0.99±0.11 | 0.96±0.18 |
| Homocysteine | ZWDET/CONT | **In Inc** | - | - |
| | ZWDET/ZW | 0.48±0.19 | - | **In Dec** |
| | ZWDET/DET | **In Inc** | - | - |
| Isoleucine | ZWDET/CONT | **0.21±0.06 ***** | 0.81±0.09 | 0.95±0.12 |
| | ZWDET/ZW | 1.05±0.28 | **2.3±0.26 *** | **2.37±0.31 *** |
| | ZWDET/DET | **0.14±0.04 ***** | 0.93±0.11 | **1.62±0.21 *** |
| Leucine | ZWDET/CONT | 0.55±0.32 | 0.7±0.47 | 0.92±0.12 |
| | ZWDET/ZW | 1.88±1.09 | 2.88±1.94 | **1.92±0.24 *** |
| | ZWDET/DET | 0.43±0.25 | 1.53±1.03 | 1.33±0.17 |
| Lysine | ZWDET/CONT | - | **In Dec** | 0.59±0.26 |
| | ZWDET/ZW | - | - | **In Inc** |
| | ZWDET/DET | - | - | **In Inc** |
| Methionine | ZWDET/CONT | **In Dec** | 0.85±0.06 | 0.68±0.13 |
| | ZWDET/ZW | - | **1.7±0.13 **** | **1.94±0.37 *** |
| | ZWDET/DET | **In Dec** | **0.45±0.03 *** | 0.69±0.13 |
| Phenylalanine | ZWDET/CONT | **0.47±0.09 **** | 1.08±0.27 | 2.41±1.68 |
| | ZWDET/ZW | **3.22±0.59 *** | **6.83±1.73 *** | **4.49±0.74 *** |
| | ZWDET/DET | **0.29±0.05 ***** | 1.22±0.31 | 1.15±0.8 |
| Proline | ZWDET/CONT | 2.84±0.76 | 1.58±0.32 | 8.16±4.19 |
| | ZWDET/ZW | **In Inc** | **7.95±1.63 *** | **In Inc** |
| | ZWDET/DET | **In Inc** | 0.61±0.12 | 15.51±7.96 |

| | | | | |
|---|---|---|---|---|
| Putrescine | ZWDET/CONT | - | **1.51±0.03 *** | 0.98±0.14 |
| | ZWDET/ZW | - | 0.56±0.01 | 0.81±0.12 |
| | ZWDET/DET | **In Dec** | **1.74±0.03 *** | 0.76±0.11 |
| Serine | ZWDET/CONT | **0.39±0.03 *** ** | 1.18±0.11 | 1.02±0.14 |
| | ZWDET/ZW | **1.65±0.14 *** | **1.97±0.19 *** | **1.9±0.27 *** |
| | ZWDET/DET | **0.37±0.03 ** ** | 1.05±0.1 | 0.99±0.14 |
| Threonine | ZWDET/CONT | **0.22±0.05 *** ** | 1.32±0.58 | 1.05±0.15 |
| | ZWDET/ZW | 1.23±0.29 | 3.08±1.34 | **2.22±0.31 *** |
| | ZWDET/DET | **0.16±0.04 *** ** | 1.13±0.49 | 1.04±0.15 |
| Valine | ZWDET/CONT | **0.34±0.09 ** ** | 1±0.11 | 1.08±0.15 |
| | ZWDET/ZW | 1.27±0.32 | **2±0.21 *** | **1.85±0.25 *** |
| | ZWDET/DET | **0.32±0.08 *** ** | **2.26±0.24 ** ** | **1.9±0.26 *** |
| **Lipid** | | | | |
| Glycero-1-C16:0 | ZWDET/CONT | **In Dec** | **In Dec** | **In Dec** |
| | ZWDET/ZW | - | - | - |
| | ZWDET/DET | - | **In Dec** | **In Dec** |
| Glycero-1-C18:0 | ZWDET/CONT | - | **In Dec** | **In Dec** |
| | ZWDET/ZW | - | - | - |
| | ZWDET/DET | - | - | **In Dec** |
| Mesaconic acid | ZWDET/CONT | 0.84±0.14 | 1.13±0.17 | 0.9±0.11 |
| | ZWDET/ZW | 0.68±0.11 | 0.88±0.13 | 0.45±0.06 |
| | ZWDET/DET | 0.87±0.14 | 0.49±0.07 | **0.53±0.06 *** |
| Palmitic acid | ZWDET/CONT | 1.73±0.85 | 1.33±0.54 | 0.6±0.39 |
| | ZWDET/ZW | 2.45±1.2 | 1.2±0.49 | 0.66±0.43 |
| | ZWDET/DET | 2.25±1.11 | 1.13±0.46 | 0.77±0.51 |
| pelargonic acid | ZWDET/CONT | **In Inc** | - | - |
| | ZWDET/ZW | 5.45±2.79 | - | - |
| | ZWDET/DET | **In Inc** | - | - |
| stearic acid | ZWDET/CONT | 1.41±0.18 | 0.75±0.1 | **In Dec** |
| | ZWDET/ZW | **2±0.26 *** | **1.57±0.21 *** | **In Dec** |
| | ZWDET/DET | 1±0.13 | 0.62±0.08 | - |
| **Organic Acid** | | | | |
| 2-ketoglutaric acid | ZWDET/CONT | **In Dec** | 2.71±1.12 | 1.65±0.27 |
| | ZWDET/ZW | - | **In Inc** | 1.76±0.29 |
| | ZWDET/DET | **In Dec** | 2±0.83 | 1.21±0.2 |
| Aconitic acid | ZWDET/CONT | 1.65±0.27 | **1.58±0.12 *** | 0.81±0.1 |
| | ZWDET/ZW | **3.64±0.6 *** | 1.66±0.13 | 1.48±0.19 |
| | ZWDET/DET | 1.44±0.24 | 1.74±0.13 | 1.28±0.16 |
| Ascorbic acid | ZWDET/CONT | **In Inc** | **In Inc** | **In Inc** |
| | ZWDET/ZW | **In Inc** | 0.69±0.65 | 0.98±0.51 |
| | ZWDET/DET | **In Inc** | **In Inc** | 1.98±1.03 |
| Aspartic acid | ZWDET/CONT | **0.53±0.12 *** | 0.99±0.07 | 1.38±0.21 |
| | ZWDET/ZW | **4.82±1.06 *** | **2.85±0.2 ** ** | **2.46±0.37 *** |
| | ZWDET/DET | **0.18±0.04 *** | 0.88±0.06 | 1.53±0.23 |

| | | | | |
|---|---|---|---|---|
| Citraconic acid | ZWDET/CONT | 0.73±0.13 | **1.39±0.05 **** | 0.94±0.14 |
| | ZWDET/ZW | 0.58±0.1 | **1.46±0.06 **** | 1.23±0.18 |
| | ZWDET/DET | 0.69±0.12 | 1.02±0.04 | 1.02±0.15 |
| Citric acid | ZWDET/CONT | 2.44±0.58 | 1.61±0.55 | 1.15±0.21 |
| | ZWDET/ZW | **5.66±1.35 *** | **3.52±0.85 *** | **2.92±0.54 *** |
| | ZWDET/DET | 2.21±0.53 | 3.24±1.1 | **3.2±0.59 *** |
| Erythronic acid | ZWDET/CONT | **6.92±1.65 *** | **1.61±0.24 *** | **1.71±0.18 *** |
| | ZWDET/ZW | **3.7±0.88 *** | 1.44±0.22 | 1.16±0.12 |
| | ZWDET/DET | **3.27±0.78 *** | 0.92±0.14 | 0.89±0.09 |
| Fumaric acid | ZWDET/CONT | 0.79±0.24 | 0.92±0.13 | **0.3±0.05 *** |
| | ZWDET/ZW | **0.46±0.14 *** | **0.62±0.09 *** | **0.27±0.04 *** |
| | ZWDET/DET | 0.63±0.19 | 0.69±0.1 | **0.34±0.06 *** |
| GABA | ZWDET/CONT | 1.52±0.45 | 1.21±0.12 | 0.97±0.14 |
| | ZWDET/ZW | 2.15±0.63 | 1.3±0.13 | 0.94±0.14 |
| | ZWDET/DET | 1.39±0.41 | **1.58±0.16 *** | 1.08±0.16 |
| Glucaric acid | ZWDET/CONT | 1.3±0.29 | 1.22±0.08 | 0.53±0.2 |
| | ZWDET/ZW | 0.46±0.1 | 0.58±0.04 | 0.38±0.14 |
| | ZWDET/DET | 1.32±0.3 | 1.42±0.09 | **0.3±0.11 **** |
| Gluconic acid | ZWDET/CONT | 1.66±0.54 | 1.31±0.47 | 0.9±0.11 |
| | ZWDET/ZW | 1.42±0.46 | 1.46±0.52 | 0.8±0.1 |
| | ZWDET/DET | 1.36±0.44 | 1.22±0.44 | **1.45±0.18 *** |
| Glucuronic acid | ZWDET/CONT | 2.55±0.45 | **2.83±0.1 **** | **1.97±0.26 *** |
| | ZWDET/ZW | **In Inc** | **4.15±0.14 ***** | **3.21±0.42 **** |
| | ZWDET/DET | - | 3.3±0.11 | 2.78±0.37 |
| Glyceric acid | ZWDET/CONT | 2.81±1.39 | 0.47±0.05 | 1.94±0.69 |
| | ZWDET/ZW | 1.08±0.53 | **0.68±0.07 *** | 0.83±0.3 |
| | ZWDET/DET | 1.23±0.61 | **0.72±0.07 *** | 0.88±0.31 |
| Itaconic acid | ZWDET/CONT | 1.27±0.22 | 1.22±0.14 | 0.99±0.11 |
| | ZWDET/ZW | 1±0.17 | 1.07±0.12 | 0.83±0.09 |
| | ZWDET/DET | 1.02±0.18 | 0.71±0.08 | 0.67±0.08 |
| Lactic acid | ZWDET/CONT | 3.33±0.87 | 0.91±0.65 | 0.35±0.06 |
| | ZWDET/ZW | 2.38±0.62 | 1.23±0.89 | 0.29±0.05 |
| | ZWDET/DET | **3.77±0.98 *** | 0.61±0.44 | 2.27±0.37 |
| Maleic acid | ZWDET/CONT | 1.46±0.23 | 0.65±0.32 | 1.18±0.13 |
| | ZWDET/ZW | 0.56±0.09 | 0.71±0.35 | 1.48±0.16 |
| | ZWDET/DET | 0.79±0.13 | 0.47±0.24 | 1.31±0.14 |
| Malic acid | ZWDET/CONT | 0.99±0.25 | 1.35±0.13 | 0.48±0.07 |
| | ZWDET/ZW | 1.3±0.32 | **1.33±0.13 *** | 0.65±0.09 |
| | ZWDET/DET | 0.88±0.22 | 1.87±0.18 | 0.66±0.09 |
| Pyroglutamic acid | ZWDET/CONT | **0.39±0.13 *** | 0.45±0.04 | 0.96±0.15 |
| | ZWDET/ZW | 1.57±0.5 | **2.8±0.23 **** | **2.54±0.41 *** |
| | ZWDET/DET | **0.31±0.1 **** | 0.22±0.02 | 1.06±0.17 |
| Succinic acid | ZWDET/CONT | **2.47±0.57 *** | 0.73±0.36 | 1.08±0.12 |
| | ZWDET/ZW | 1.79±0.41 | 1.3±0.65 | 0.75±0.08 |
| | ZWDET/DET | 1.61±0.37 | 0.74±0.37 | 0.9±0.1 |

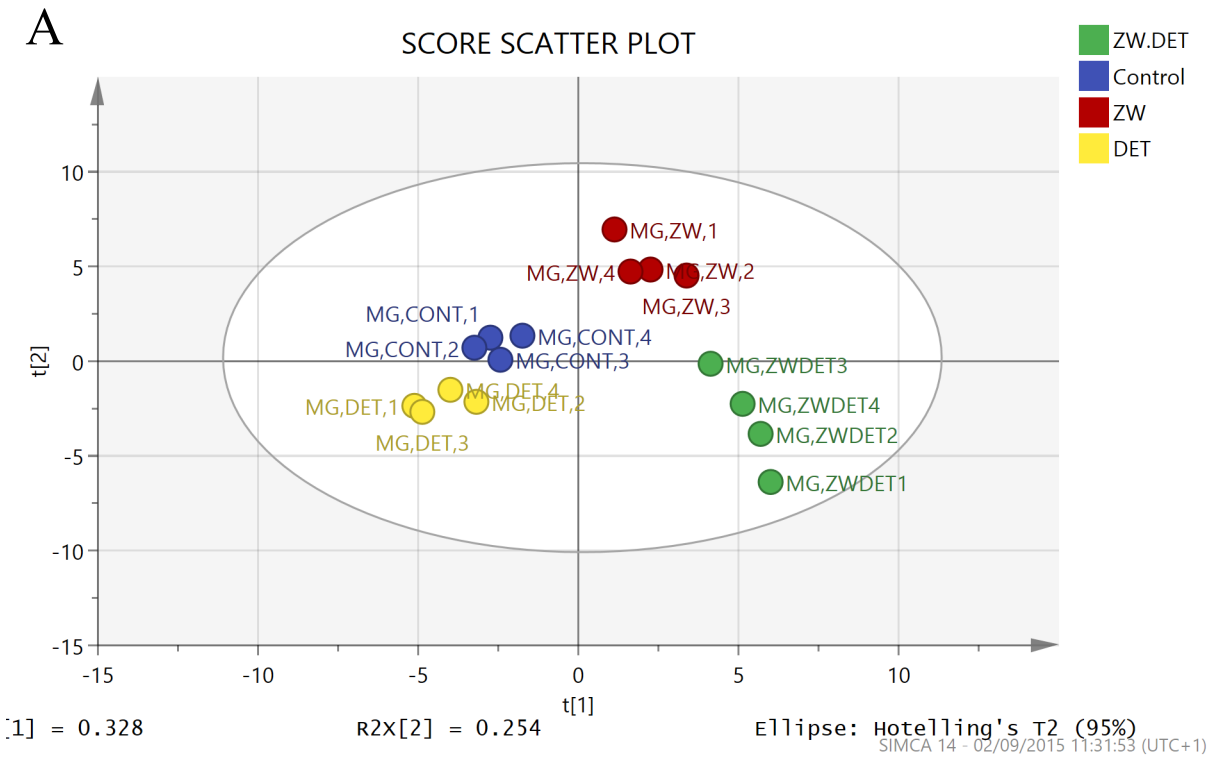
| | | | | |
|---|---|---|---|---|
| **Phosphate** | | | | |
| Glucose-6-Phosphate | ZWDET/CONT | - | 2.22±0.28 | 1.06±0.25 |
| | ZWDET/ZW | - | **In Inc** | **In Inc** |
| | ZWDET/DET | - | **In Inc** | **In Inc** |
| Glycerol-3-Phosphate | ZWDET/CONT | 2.89±0.66 | **2.61±0.21 **** | 0.82±0.11 |
| | ZWDET/ZW | **7.44±1.7 *** | **2.7±0.21 **** | 0.73±0.1 |
| | ZWDET/DET | 1.47±0.34 | **2.11±0.17 *** | 1.51±0.2 |
| Inositol-6-Phosphate | ZWDET/CONT | 1.33±0.33 | 1.22±0.08 | **3±0.45 *** |
| | ZWDET/ZW | **In Inc** | **3.54±0.22 ***** | 2.02±0.3 |
| | ZWDET/DET | 1.2±0.3 | 1.55±0.1 | **2.54±0.38 *** |
| Phosphate | ZWDET/CONT | 1.14±0.29 | 1.63±0.35 | 1.96±1.04 |
| | ZWDET/ZW | 1.72±0.44 | 1.43±0.31 | 2.51±1.33 |
| | ZWDET/DET | 1.38±0.35 | 1.21±0.26 | 1.84±0.98 |
| **Polyol** | | | | |
| Glycerol | ZWDET/CONT | 1.63±0.44 | 0.85±0.19 | 0.81±0.1 |
| | ZWDET/ZW | 1.03±0.28 | 1.71±0.39 | 0.55±0.07 |
| | ZWDET/DET | 1.98±0.54 | 0.94±0.21 | 0.77±0.1 |
| Inositol | ZWDET/CONT | 1.54±0.42 | 1.64±0.48 | 0.81±0.12 |
| | ZWDET/ZW | 1.35±0.37 | 2.9±0.85 | 1.06±0.15 |
| | ZWDET/DET | 1.76±0.48 | 1.7±0.5 | 1.03±0.15 |
| **Sugar** | | | | |
| Arabinose/ribose/xylose | ZWDET/CONT | 0.87±0.47 | 0.77±0.12 | 0.81±0.17 |
| | ZWDET/ZW | 1.07±0.58 | 1.05±0.17 | 0.79±0.17 |
| | ZWDET/DET | 0.71±0.39 | 0.84±0.14 | 0.93±0.2 |
| Erythrose | ZWDET/CONT | **In Inc** | 1.12±0.41 | **In Inc** |
| | ZWDET/ZW | 0.29±0.02 | 1.53±0.56 | 0.57±0.22 |
| | ZWDET/DET | 0.62±0.04 | **In Inc** | 1.46±0.56 |
| Fructose | ZWDET/CONT | 0.82±0.65 | 0.74±0.03 | 0.95±0.09 |
| | ZWDET/ZW | 1.19±0.94 | 1.16±0.05 | 0.98±0.09 |
| | ZWDET/DET | 0.64±0.5 | **0.89±0.04 *** | 0.94±0.09 |
| Glucose | ZWDET/CONT | 1±0.47 | 1.12±0.27 | 0.75±0.17 |
| | ZWDET/ZW | 1.13±0.53 | 1.55±0.37 | 1.36±0.31 |
| | ZWDET/DET | 0.71±0.33 | 1.13±0.27 | 1.02±0.23 |
| Me-Fructose | ZWDET/CONT | **In Dec** | 0.25±0.01 | 0.59±0.06 |
| | ZWDET/ZW | - | 0.26±0.01 | 1.2±0.12 |
| | ZWDET/DET | **In Dec** | 0.12±0.01 | **0.5±0.05 *** |
| Sucrose | ZWDET/CONT | **2.84±0.7 *** | **2.14±0.14 **** | 1.17±0.39 |
| | ZWDET/ZW | **4.66±1.15 *** | 1.85±0.12 | 1.28±0.42 |
| | ZWDET/DET | 2.46±0.61 | **2.54±0.16 **** | 3.86±1.28 |
| Trehalose | ZWDET/CONT | - | **In Dec** | 3.3±1.44 |
| | ZWDET/ZW | - | - | **In Inc** |
| | ZWDET/DET | - | - | **In Inc** |
| Xylulose | ZWDET/CONT | **0.24±0.12 **** | 1.02±0.16 | - |
| | ZWDET/ZW | 0.56±0.28 | 1.3±0.2 | - |
| | ZWDET/DET | **0.2±0.1 **** | 0.9±0.14 | - |

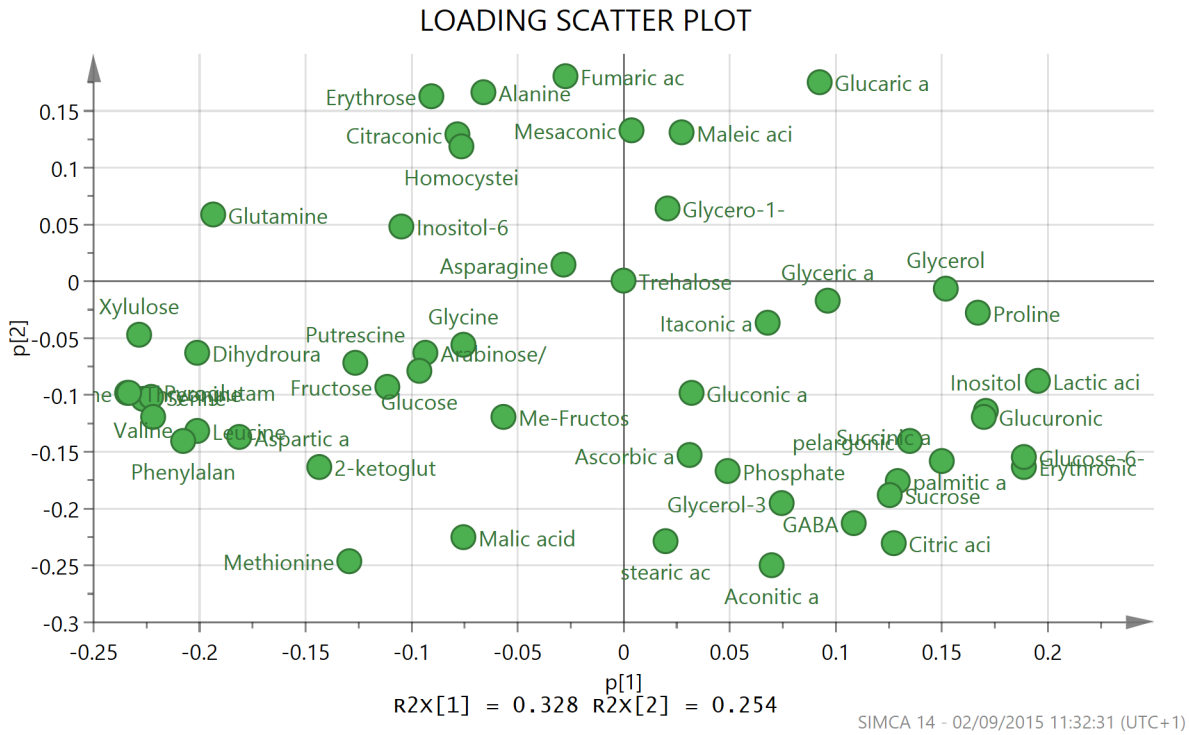
The datasets obtained from the GC-MS processed data was analysed by PCA. The PCA outputs used were plots of the score and loading parameters. The score plots were performed to illustrate the overall variance of chemical composition between lines and the loadings plot the contribution of each metabolite to the overall variance respectively. PCA was performed for 3 different stages of fruit ripening in CrtZW.DET1 and its derived parental and background lines (Control (T56.MM), CrtZW (T56.MM) and DET1 (T56.MM)) (see Figure 4-16).

At the mature green stage the score values of the scatter plots displayed significant different clusters for each genotype. CrtZW and CrtZW.DET1 lines clustered considerably apart from each other, which indicates the differences between them. The control and DET1 genotypes cluster closer to each other. In the loading scatter plot the distribution of metabolites had two slight clusters of compound on right and left hand side of loading plot as a result of CrtZW.DET1 and DET1 samples respectively. The altered compounds at MG stage that contributed to the clustering of CrtZW.DET1 samples on the right hand side of loading plots were mainly organic acids. For instance glucuronic acid, lactic acid and erythronic acid can be mentioned. Considering that most of changes occurred at 3 dpb, this set of data also showed a good separation at 3 dpb as well, with the control line clustering in the middle of scatter plot and grouping of other lines around it. In 3dpb stage CrtZW and CrtZW.DET1 were distinctly grouped far from each other similar to MG stage. A considerable shift of metabolites to right panel was observed which was in result of altered metabolites in CrtZW.DET1 line; mainly consist of organic acids (for instance glucuronic acid, citric acid) and some of amino acids and phosphates like valine and glycerol-3-phosphate respectively (see Figure 4-16). At ripe stage Control and DET1 lines clustered close to each other in the centre of scatter plots, while CrtZW and CrtZW.DET1 grouped separately in two different directions. In the loading plot the metabolites distributed on the right hand side similar to 3 dpb, suggesting a specific chemical composition effect on CrtZW.DET1 at this stage (see Figure 4-16). This chemicals affecting CrtZW.DET1 at this stage are mainly amino acids for example glutamine, leucine, isoleucine, serin, valine and etc.

In general, the 4 lines indicated a good model of clustering in all of stages representing their differences at each stages of ripening.

A


SCORE SCATTER PLOT
ZW.DET
Control
ZW
DET
MG,ZW,1
MG,ZW,4
MG,ZW,2
MG,ZW,3
MG,CONT,1
MG,CONT,2
MG,CONT,4
MG,CONT,3
MG,ZWDET3
MG,DET,4
MG,DET,1
MG,DET,2
MG,ZWDET4
MG,ZWDET2
MG,DET,3
MG,ZWDET1
t[2]
t[1]
1] = 0.328
R2X[2] = 0.254
Ellipse: Hotelling's T2 (95%)
SIMCA 14 - 02/09/2015 11:31:53 (UTC+1)


LOADING SCATTER PLOT
Fumaric ac
Glucaric a
Erythrose
Alanine
Citraconic
Mesaconic
Maleic aci
Homocystei
Glutamine
Glycero-1-
Inositol-6
Asparagine
Trehalose
Glyceric a
Glycerol
Xylulose
Proline
Itaconic a
Putrescine
Glycine
Dihydroura
Arabinose/
Fructose
Glucose
Gluconic a
Inositol
Lactic aci
Glucuronic
Me-Fructos
Valine
Aspartic a
pelargonic a
Succinic a
Ascorbic a
Glucose-6-
Phosphate
Erythronic
Phenylalan
2-ketoglut
palmitic a
Sucrose
Glycerol-3
GABA
Malic acid
Citric aci
Methionine
stearic ac
Aconitic a
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p[1]
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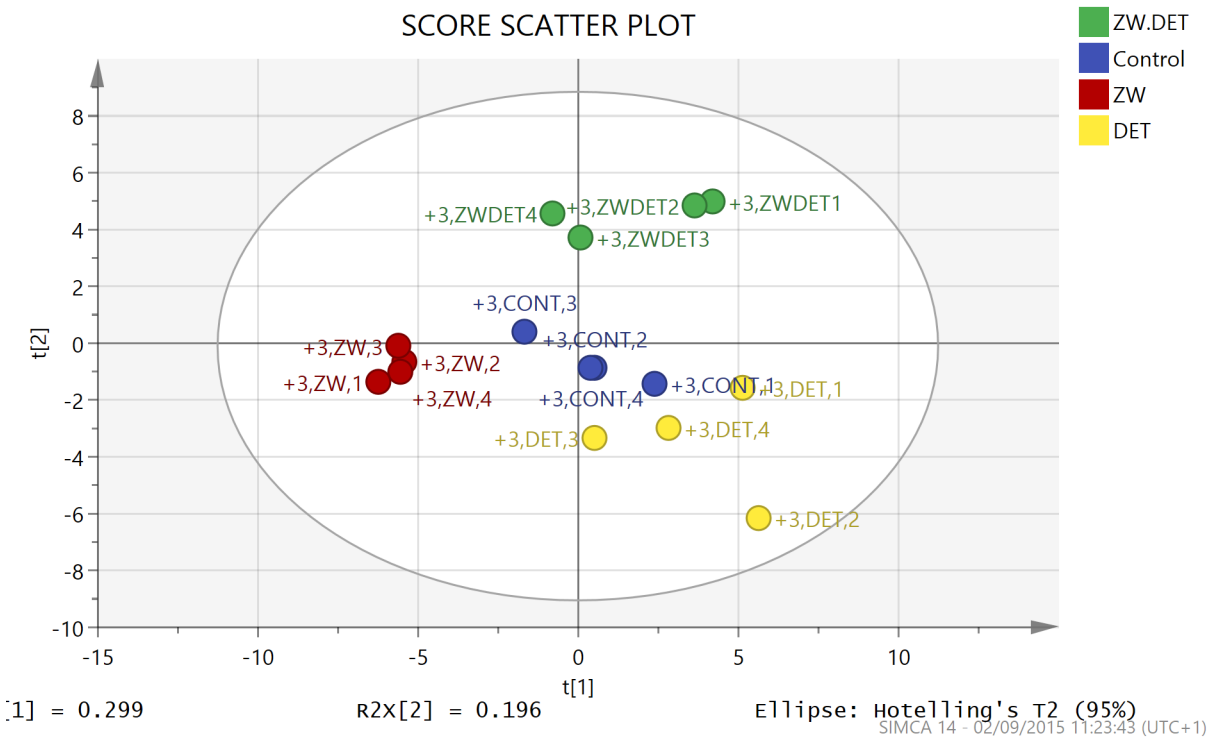
B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Control
ZW
DET
+3,ZWDET4
+3,ZWDET2
+3,ZWDET1
+3,ZWDET3
+3,CONT,3
+3,CONT,2
+3,ZW,3
+3,ZW,2
+3,ZW,1
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+3,DET,2
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Ellipse: Hotelling's T2 (95%)
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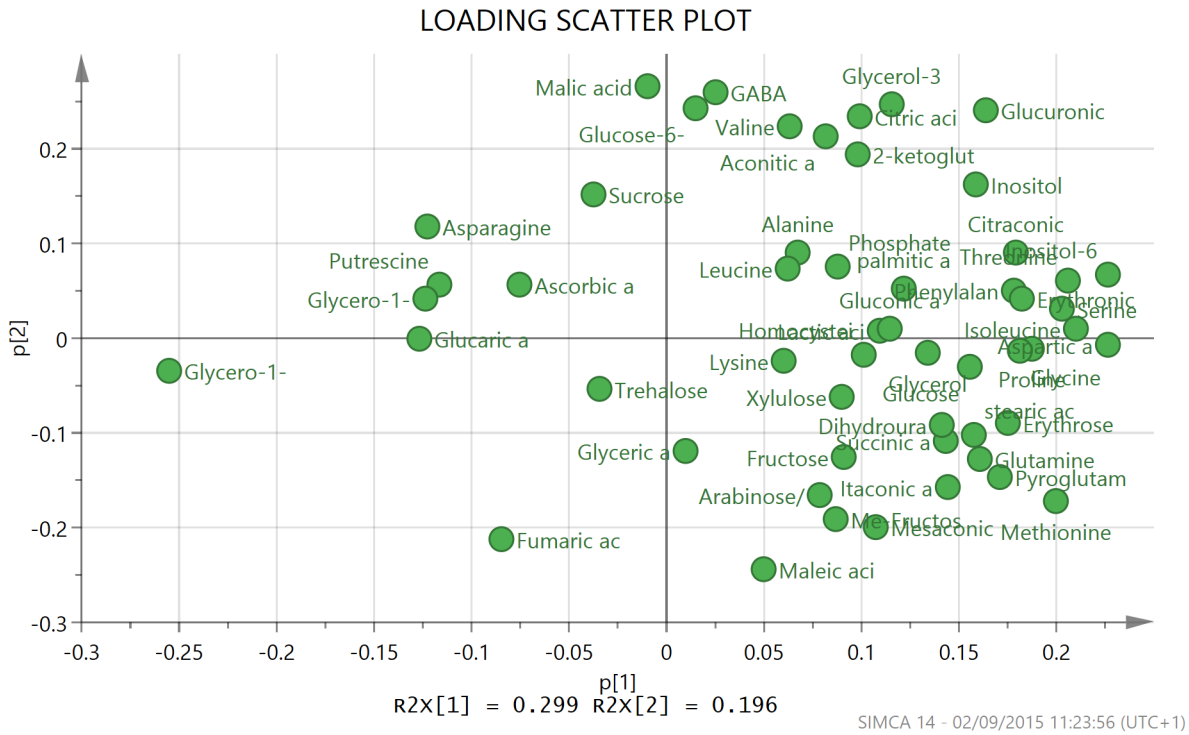
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Malic acid
GABA
Glycerol-3
Glucose-6-
Valine
Citric aci
Glucuronic
Aconitic a
2-ketoglut
Inositol
Sucrose
Asparagine
Alanine
Citraconic
Phosphate
palmitic a
Putrescine
Leucine
Glycero-1-
Ascorbic a
Gluconic a
Glucaric a
Lysine
Glycero-1-
Trehalose
Xylulose
Glycerol
Glucose
Dihydroura
Erythrose
Glyceric a
Fructose
Glutamine
Pyroglutam
Arabinose/
Itaconic a
Fumaric ac
Mesaconic
Methionine
Maleic aci
p[2]
p[1]
R2X[1] = 0.299 R2X[2] = 0.196
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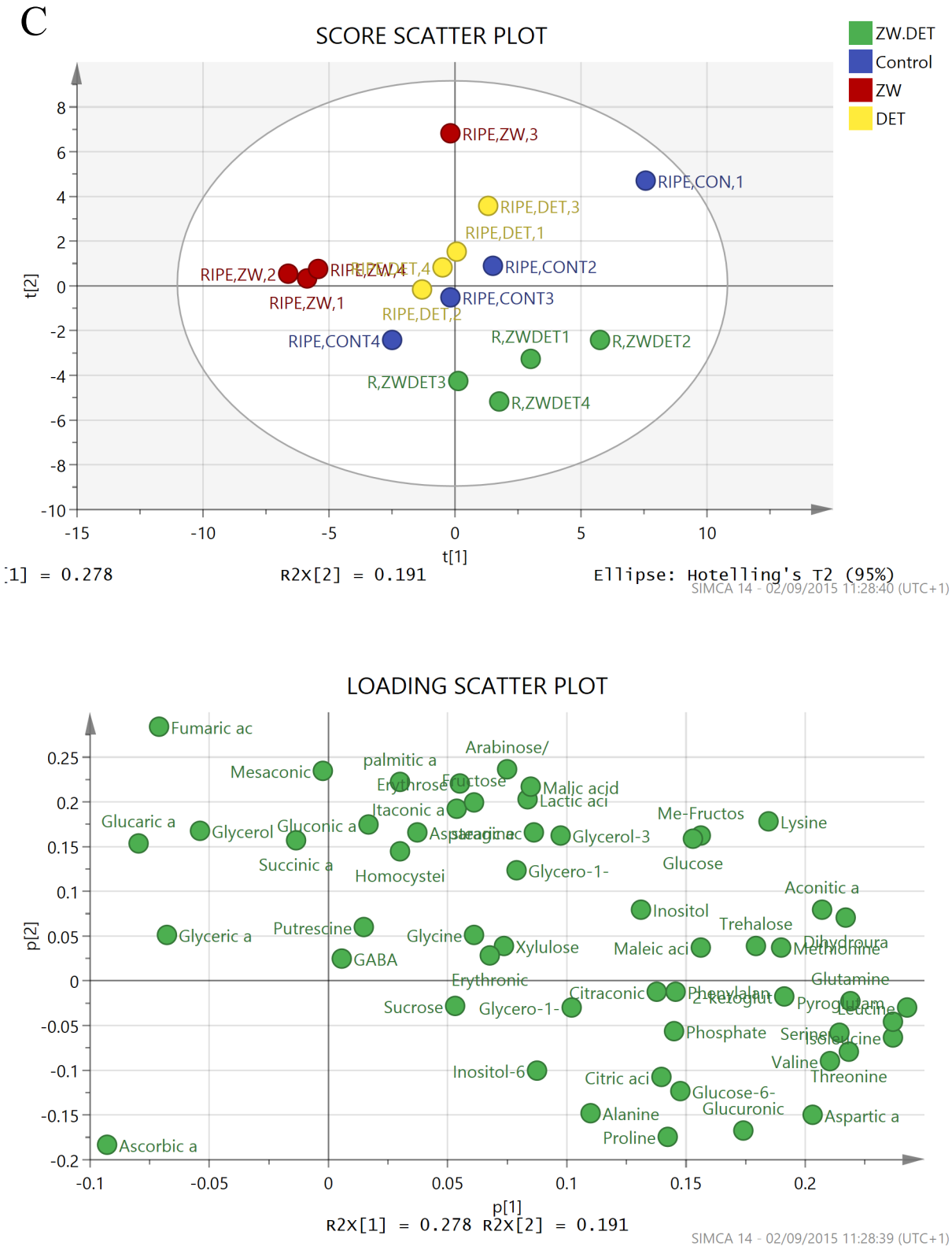
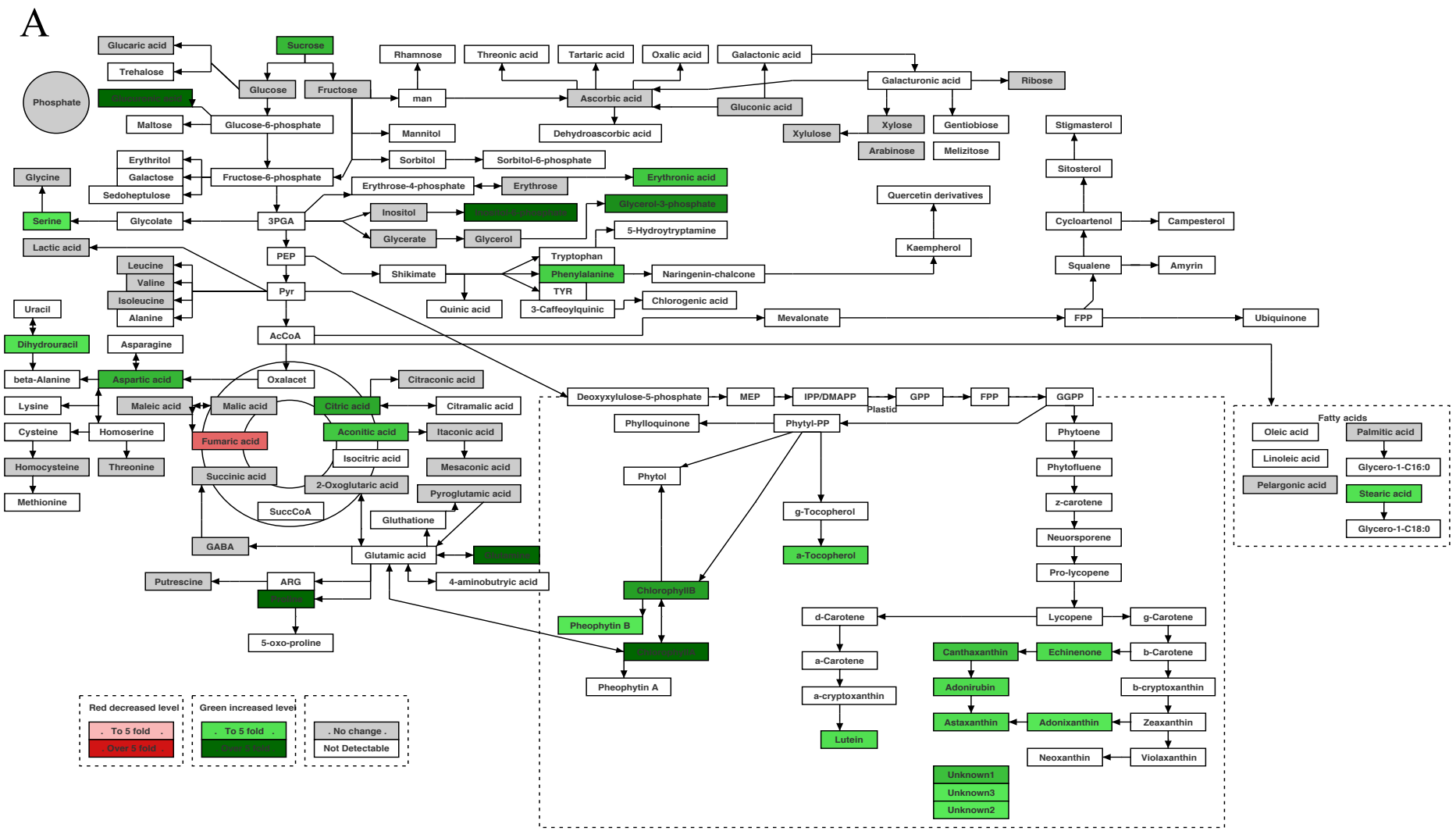



Figure 4-16 Principal component analysis (PCA) of ZWDET, CrtZW, DET1 AND Control second generation tomato fruits at 3 different stages of ripening. The metabolites represented were detected by GC-MS.

Score scatter plots and loading scatter plots are illustrated for (A) Mature green stage, (B) 3 days post breaker and (C) ripe stages. These subsequent stages of ripening are shown as MG, +3 and RIPE (R) respectively in score scatter plots. The full names of the variables (metabolites) on the loading scatter

plots are available in Table 4-16. Four biological with minimum three technical replicates were analysed. The extraction method, analytical and data processing method used to analyse polar metabolites are described in sections 2.2.1.2, 2.2.1.3, 2.2.4 and 2.10.

For a better visual comparison about the metabolite alterations in fruits at each stage, the changes were represented in biochemical pathways (Figure 4-17). The changes are illustrated as colour change reflecting the fold changes of metabolite ratio in CrtZW.DET1 to CrtZW parental line. These visual figures help for a better understanding of the overall changes occurring at different stages of ripening (see section 4.7.1.8).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Rhamnose
Threonic acid
Tartaric acid
Oxalic acid
Galactonic acid
Trehalose
Phosphate
Glucose
Fructose
man
Ascorbic acid
Galacturonic acid
Ribose
Maltose
Glucose-6-phosphate
Gluconic acid
Xylose
Gentiobiose
Mannitol
Dehydroascorbic acid
Xylulose
Arabinose
Melizitose
Erythritol
Sorbitol
Sorbitol-6-phosphate
Stigmasterol
Glycine
Galactose
Fructose-6-phosphate
Erythrose-4-phosphate
Erythrose
Erythronic acid
Sitosterol
Sedoheptulose
Inositol
Glycerol-3-phosphate
Quercetin derivatives
Serine
Glycolate
3PGA
Glycerate
Glycerol
5-Hydroxytryptamine
Cycloartenol
Campesterol
Lactic acid
PEP
Tryptophan
Kaempherol
Leucine
Shikimate
Phenylalanine
Naringenin-chalcone
Squalene
Amyrin
Valine
Pyr
TYR
Isoleucine
Quinic acid
3-Caffeoylquinic
Chlorogenic acid
Uracil
Alanine
Mevalonate
FPP
Ubiquinone
Dihydrouracil
Asparagine
AcCoA
beta-Alanine
Aspartic acid
Oxalacet
Citraconic acid
Deoxyxylulose-5-phosphate
MEP
IPP/DMAPP
GPP
FPP
GGPP
Lysine
Maleic acid
Malic acid
Citric acid
Citramalic acid
Plastid
Fatty acids
Phylloquinone
Phytyl-PP
Cysteine
Homoserine
Fumaric acid
Aconitic acid
Itaconic acid
Oleic acid
Palmitic acid
Phytoene
Linoleic acid
Isocitric acid
Phytol
Phytofluene
Pelargonic acid
Glycero-1-C16:0
Homocysteine
Threonine
Mesaconic acid
Stearic acid
Succinic acid
2-Oxoglutaric acid
g-Tocopherol
z-carotene
Glycero-1-C18:0
Pyroglutamic acid
Methionine
SuccCoA
Gluthatione
a-Tocopherol
Neuorsporene
GABA
Glutamic acid
Pro-lycopene
Putrescine
ARG
4-aminobutryic acid
ChlorophyllB
d-Carotene
Lycopene
g-Carotene
Pheophytin B
Canthaxanthin
Echinenone
b-Carotene
a-Carotene
Adonirubin
b-cryptoxanthin
5-oxo-proline
Pheophytin A
a-cryptoxanthin
Astaxanthin
Adonixanthin
Zeaxanthin
Lutein
Neoxanthin
Violaxanthin
Unknown1
Unknown3
Unknown2
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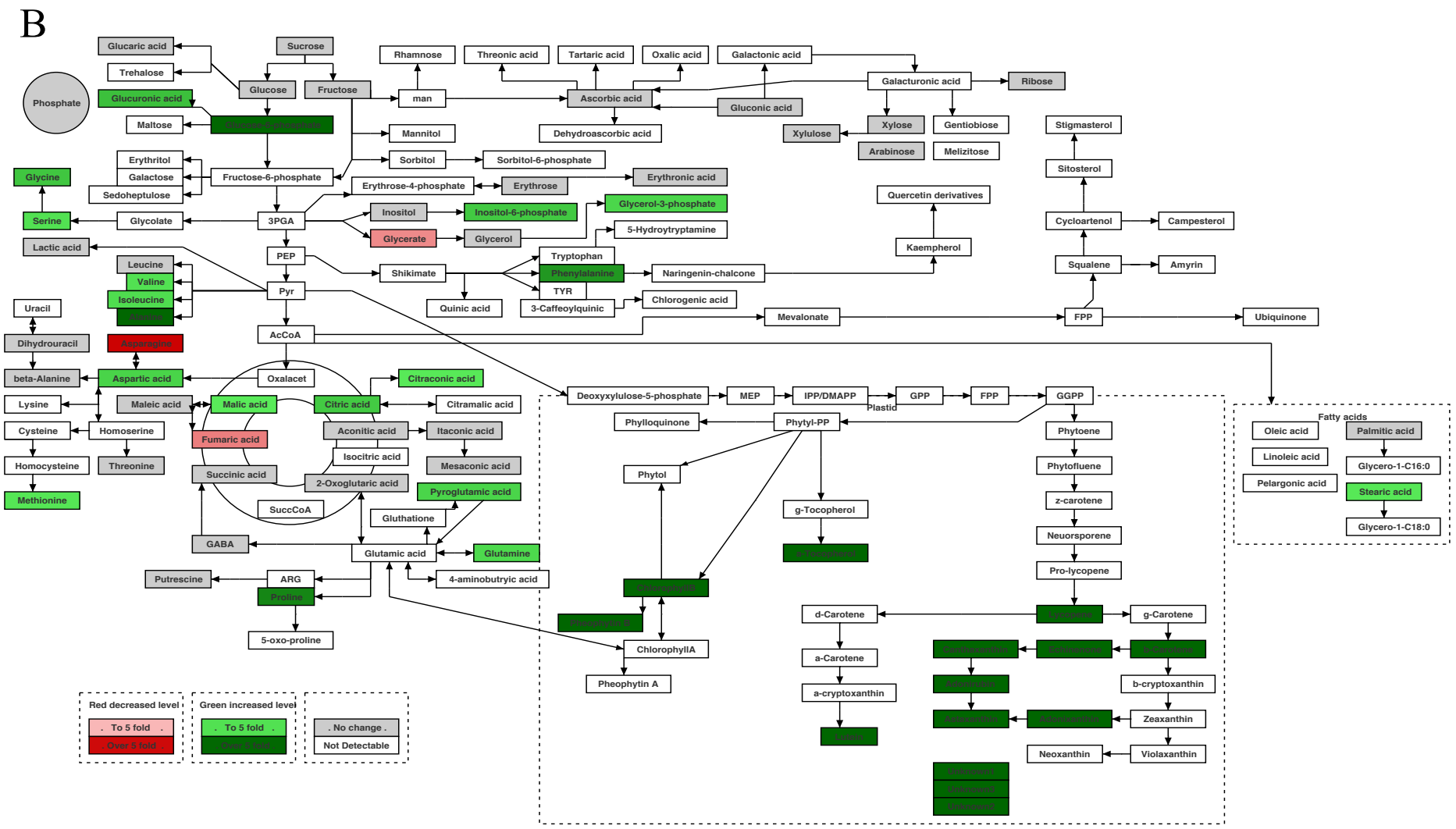
. To 5 fold .
. Over 5 fold
Green increased level
. To 5 fold .
. Over 5 fold
. No change .
Not Detectable


B
Glucaric acid
Sucrose
Rhamnose
Threonic acid
Tartaric acid
Oxalic acid
Galactonic acid
Trehalose
Glucose
Fructose
Galacturonic acid
Ribose
Phosphate
Glucuronic acid
man
Ascorbic acid
Gluconic acid
Maltose
Mannitol
Dehydroascorbic acid
Xylose
Gentiobiose
Xylulose
Arabinose
Melizitose
Stigmasterol
Erythritol
Sorbitol
Sorbitol-6-phosphate
Sitosterol
Glycine
Galactose
Fructose-6-phosphate
Erythrose-4-phosphate
Erythrose
Erythronic acid
Sedoheptulose
Quercetin derivatives
Inositol
Inositol-6-phosphate
Glycerol-3-phosphate
Cycloartenol
Campesterol
Serine
Glycolate
3PGA
Glycerate
Glycerol
5-Hydroxytryptamine
Lactic acid
Kaempherol
Squalene
Amyrin
PEP
Tryptophan
Leucine
Shikimate
Phenylalanine
Naringenin-chalcone
Valine
Pyr
TYR
Isoleucine
Quinic acid
3-Caffeoylquinic
Chlorogenic acid
Uracil
Mevalonate
FPP
Ubiquinone
AcCoA
Dihydrouracil
Asparagine
Deoxyxylulose-5-phosphate
MEP
IPP/DMAPP
GPP
FPP
GGPP
Plastid
Fatty acids
beta-Alanine
Aspartic acid
Oxalacet
Citraconic acid
Oleic acid
Palmitic acid
Lysine
Maleic acid
Malic acid
Citric acid
Citramalic acid
Phylloquinone
Phytyl-PP
Linoleic acid
Glycero-1-C16:0
Cysteine
Homoserine
Fumaric acid
Aconitic acid
Itaconic acid
Phytoene
Pelargonic acid
Stearic acid
Isocitric acid
Phytol
Phytofluene
Homocysteine
Threonine
Succinic acid
2-Oxoglutaric acid
Mesaconic acid
z-carotene
Glycero-1-C18:0
Pyroglutamic acid
g-Tocopherol
Neuorsporene
Methionine
SuccCoA
Gluthatione
Pro-lycopene
GABA
Glutamic acid
Glutamine
Chlorophyll B
d-Carotene
g-Carotene
Putrescine
ARG
4-aminobutryic acid
Proline
Chlorophyll A
a-Carotene
b-cryptoxanthin
5-oxo-proline
ChlorophyllA
a-cryptoxanthin
Zeaxanthin
Pheophytin A
Neoxanthin
Violaxanthin
Red decreased level
. To 5 fold .
. Over 5 fold
Green increased level
. To 5 fold .
. No change .
Not Detectable

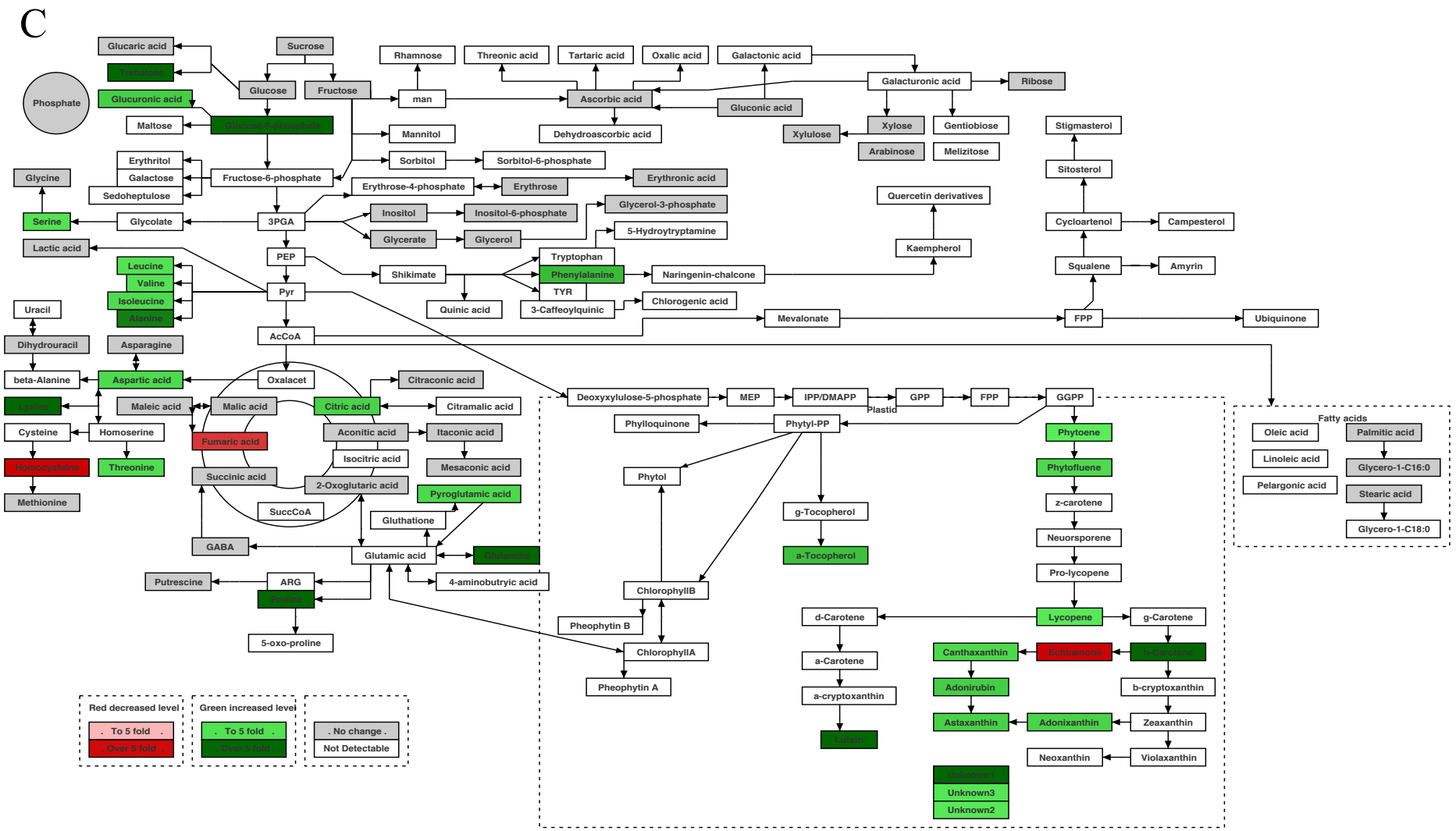



Figure 4-17 Pathway displays of metabolite changes in ZW.DET1 compared ZW line at different stages of fruit development and ripening

Figure 4-17 Pathway displays of metabolite changes in ZW.DET1 compared ZW line at different stages of fruit development and ripening

Pathway displays of metabolite changes are illustrated in (A) Mature green stage, (B) 3 days post breaker, (C) 7 days post breaker. The figures displayed metabolite changes quantitatively over schematic representations in biochemical pathway using BioSynLab software (www.biosynlab.com). The fold changes of compound levels are presented as ratio of ZW.DET1 level to the ZW comparator. Light red indicates up to 5 folds decrease and dark red more than 5 fold decrease whereas light green illustrates up to 5 folds increase and dark green more than 5 fold increase. Grey shows no significant changes. White indicates that the metabolite was undetectable either in the samples or in the analytical platforms used. 3PGA, glyceraldehyde-3-phosphate; Ac-CoA, acetyl-coenzyme A; ARG, arginine; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl-pyrophosphate; GPP, geranyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; SuccCoA, succinyl-coenzyme A; TYR, tyrosine.

### 4.2.6 Metabolite profile changes during fruit development and ripening in CrtZW.APRR2 and control.

To understand about the other metabolites changes among F2 generation of CrtZW.APRR2 line and its derived F2 control (Mic.MM) line, GC MS metabolite profiling was carried out (see section 2.2.4). Following quantification of metabolites statistical analyses was performed as explained in section 2.10. Around 50 metabolites were identified and quantified in 3 stages of development and ripening in CrtZW.APRR2 line and control. As the CrtZW.APRR2 line did not show a considerable improvement in carotenoids and ketocarotenoids to its derived lines by UPLD-PDA analysis (see section 4.2.4.2). Therefore, the GC data performed just for Control (Mic.MM) line. The analysed samples at 3dpb and ripe in CrtZW.APRR2 line were represent 3 Phen and 7 Phen as described in section 4.2.2.3. As it was expected, considerable changes were not observed in the metabolites level to control. At MG and ripe stages just 4 and 7 metabolites showed a significant change in the CrtZW.APRR2 to control respectively while at 3dpb more changes in metabolites were detected. Overall, in CrtZW.APRR2 lines most of altered metabolites at 3dpb and ripe represented a decrease in comparison to control.

Many of amino acids (alanine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine and etc.) and some of organic acids (aspartic acid and pyroglutamic acid) decreased at 3dpb but fumaric acid and isocitric acid from TCA cycle increased.

At ripe stage 4 of amino acids decreased and just fumaric acid from organic acids group and inositol-6-Phosphate from phosphate group presented an indefinite increase. In general, CrtZW.APRR2 did not make a considerable difference to control line and the overall trend of compound changes was a decrease trend. (see Table 4-17) and section 4.7.2 for conclusion.

Table 4-17 The ratios of metabolite changes at different stages of fruit development and ripening in the F2 CrtZW.APRR2 line tomato fruits compared to its derived Control (Mic.MM) line.

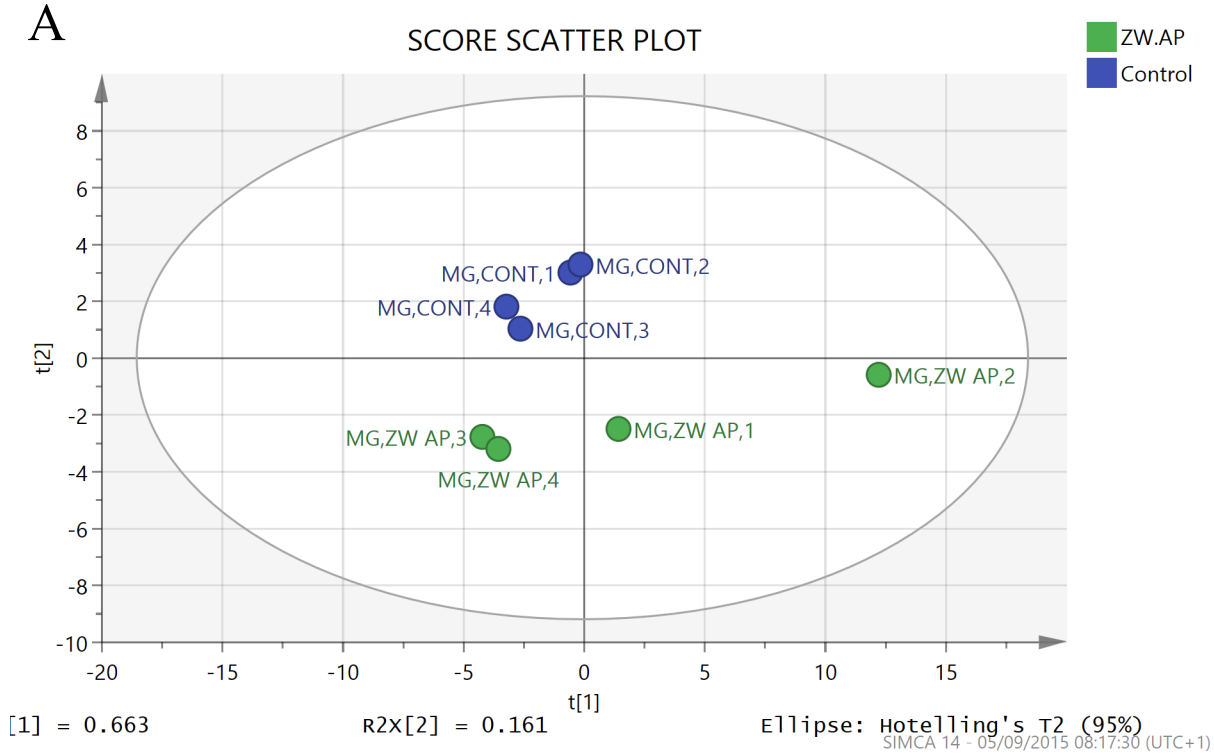
Data obtained from GC-MS analytical platform with subsequent statistical analysis. The data are presented as mean ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of P<0.05, P<0.01 and P<0.001 are shown by *, ** and *** respectively. If a metabolite is unique to CrtZW.APRR2 at the concentration used is shown as In Inc (indefinite increase); and when is unique to a Control line at the sample concentration used is shown as In Dec (indefinite decrease) and "-" indicates metabolite was not detectable in both CrtZW.APRR2 and the comparator at the sample concentration used. GABA: γ-Aminobutyric acid.

| | ZW.APRR2/Control (Mic.MM) Ratio | | |
|---|---|---|---|
| **Metabolite** | **MG** | **+3** | **Ripe** |
| **Amion Acid** | | | |
| Alanine | 3.64±1.82 | - | **0.2±0.2 *** |
| Asparagine | **In Dec** | **0.18±0.02 *** ** | 0.28±0.04 |
| Beta-Alanine | - | 2.58±0.18 | - |
| Cysteine | - | **In Dec** | **0.28±0.02 *** |
| Dihydrouracil | 0.62±0.28 | **0.58±0.09 **** | 0.5±0.03 |
| Glutamine | 1.25±0.55 | 0.56±0.27 | 0.37±0.04 |
| Glycine | 1.14±0.62 | **0.48±0.18 *** | 1.31±0.3 |
| Isoleucine | 1.49±0.66 | **0.61±0.06 **** | **0.32±0 *** |
| Leucine | 1.84±0.82 | **0.62±0.06 **** | 0.43±0.01 |
| Lysine | - | **In Dec** | 0.18±0.01 |
| Methionine | 0.87±0.39 | **0.42±0.04 *** ** | 0.47±0.04 |
| Phenylalanine | 1.7±0.81 | 0.52±0.05 | 0.93±0.29 |
| Proline | 16.1±6.93 | **0.21±0 *** | 0.51±0 |
| Putrescine | 0.85±0.28 | 0.23±0.01 | 0.5±0.01 |
| Serine | 1.67±1.28 | 0.84±0.07 | **0.38±0.01 *** |
| Threonine | 1.13±0.5 | 0.72±0.29 | **0.34±0.01 *** |
| Valine | 1.32±0.57 | **0.69±0.07 *** | 0.43±0.01 |
| **Lipid** | | | |
| Mesaconic acid | 1.81±0.96 | 0.97±0.22 | 0.89±0.05 |
| Palmitic acid | 6.45±0.39 | **In Inc** | 0.34±0.18 |
| **Organic Acid** | | | |
| 2-ketoglutaric acid | - | **In Inc** | - |
| Aconitic acid | 0.85±0.42 | 0.67±0.11 | 1.06±0.31 |
| Aspartic acid | 1.24±0.58 | **0.45±0.05 *** ** | 0.66±0.02 |
| Citraconic acid | 1.17±0.49 | 0.96±0.27 | 0.87±0.06 |
| Citric acid | 1.69±1.32 | 1.72±0.78 | 1.14±0.4 |
| Erythronic acid | **In Inc** | 0.38±0.05 | 1.66±0.43 |
| Fumaric acid | 1.44±0.58 | **1.63±0.12 **** | 1.07±0.12 |
| GABA | 1.44±0.61 | 1.05±0.06 | 0.84±0.04 |
| Glucaric acid | 3.78±3.04 | 0.32±0.06 | 0.36±0.12 |
| Gluconic acid | 1.75±0.84 | 1.1±0.22 | 0.8±0.25 |
| Glyceric acid | 0.91±0.42 | 0.54±0.31 | 0.71±0.23 |
| Isocitric acid | 1.08±0.53 | **In Inc** | **In Inc** |
| Itaconic acid | 1.13±0.49 | 0.99±0.23 | 0.93±0.05 |
| Lactic acid | **1.83±0.2 *** | 0.81±0.14 | 0.17±0.07 |

| | | | |
|---|---|---|---|
| Maleic acid | 1.04±0.32 | 2.37±0.89 | 0.93±0.4 |
| Malic acid | 1.33±0.59 | 1.22±0.13 | 1.02±0.03 |
| Pyroglutamic acid | 0.5±0.22 | **0.4±0.06 **** | 0.61±0.01 |
| Succinic acid | 2.18±1.59 | 0.85±0.15 | 1.05±0.13 |
| **Phosphate** | | | |
| Glycerol-3-Phosphate | 1.28±0.69 | 0.98±0.17 | 1.01±0.03 |
| Inositol-6-Phosphate | - | - | **In Inc** |
| Phosphate | 1.12±0.49 | 0.6±0.07 | 0.57±0.02 |
| **Polyol** | | | |
| Glycerol | 2.15±0.59 | 0.76±0.03 | 0.77±0.1 |
| Inositol | 4.98±2.62 | 1.22±0.48 | 1.07±0.33 |
| **Sugar** | | | |
| Arabinose/ribose/xylose | 1.65±0.94 | 1.19±0.3 | 1.01±0.25 |
| Fructose | 0.97±0.42 | 1.02±0.11 | 0.84±0.08 |
| Glucose | 1.31±0.67 | 1.33±0.07 | 0.97±0.27 |
| Me-Fructose | 0.32±0.14 | 0.86±0.26 | 0.7±0.01 |
| Sucrose | 8.02±4.31 | **In Inc** | - |
| Xylulose | **In Inc** | **In Inc** | 0.74±0.07 |

The GC analysis data was used for creating multivariate principal component analysis (PCA) for this set of data as well. PCA presented as score scatter plots illustrating the overall variance of chemical composition between CrtZW.APRR2 and Control (Mic.MM) lines, and loading scatter plots representing contribution of each metabolite to the overall variance. PCA was performed for 3 different stages of fruit ripening in CrtZW.APRR2 and its derived background Control (Mic.MM) line (see Figure 4-18). In mature green stage scatter plots of the score values showed distinctly different groups for each lines of CrtZW.APRR2 and the Control (Mic.MM), which represent the differences between them. A considerable shift of all types of metabolites observed to the right hand side of loading plot, which was in result of shift of one of CrtZW.APRR2 replicates to the right. At 3dpb stage the samples separated clearly based on just 1 component on the score scatter plot. Despite having most of changes at this stage, in the loading scatter plot the distribution of metabolites were nearly even throughout the plot, suggesting equal effect of these metabolites on both lines at 3 dpb. The difference of clustering was also visible at ripe stage similar to previous stages. The loading scatter plot indicated a shift to the right hand side in due of one of control replicates' shift to the right side. This shift was a combination of all types of metabolites (see Figure 4-18). In general, the lines indicate a good model of clustering in all of stages confirming their differences at each stages of ripening.

A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Control
MG,CONT,1
MG,CONT,2
MG,CONT,4
MG,CONT,3
MG,ZW AP,2
MG,ZW AP,3
MG,ZW AP,1
MG,ZW AP,4
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t[1]
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R2X[2] = 0.161
Ellipse: Hotelling's T2 (95%)
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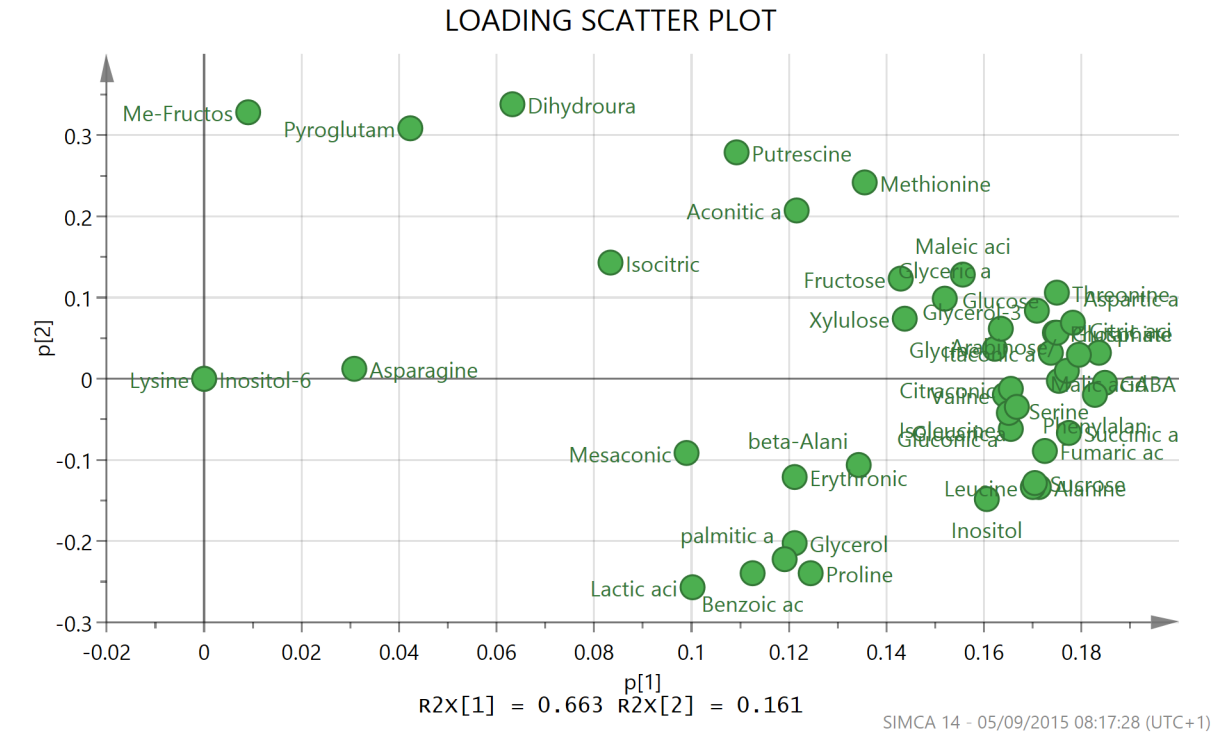
LOADING SCATTER PLOT
Me-Fructos
Pyroglutam
Dihydroura
Putrescine
Methionine
Aconitic a
Isocitric
Maleic aci
Fructose
Glucose
Threonine
Xylulose
Glycerol-3
Lysine
Inositol-6
Asparagine
Citraconic
Serine
Mesaconic
beta-Alani
Succinic a
Fumaric ac
Erythronic
Leucine
Alanine
Sucrose
Inositol
palmitic a
Glycerol
Proline
Lactic aci
Benzoic ac
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p[1]
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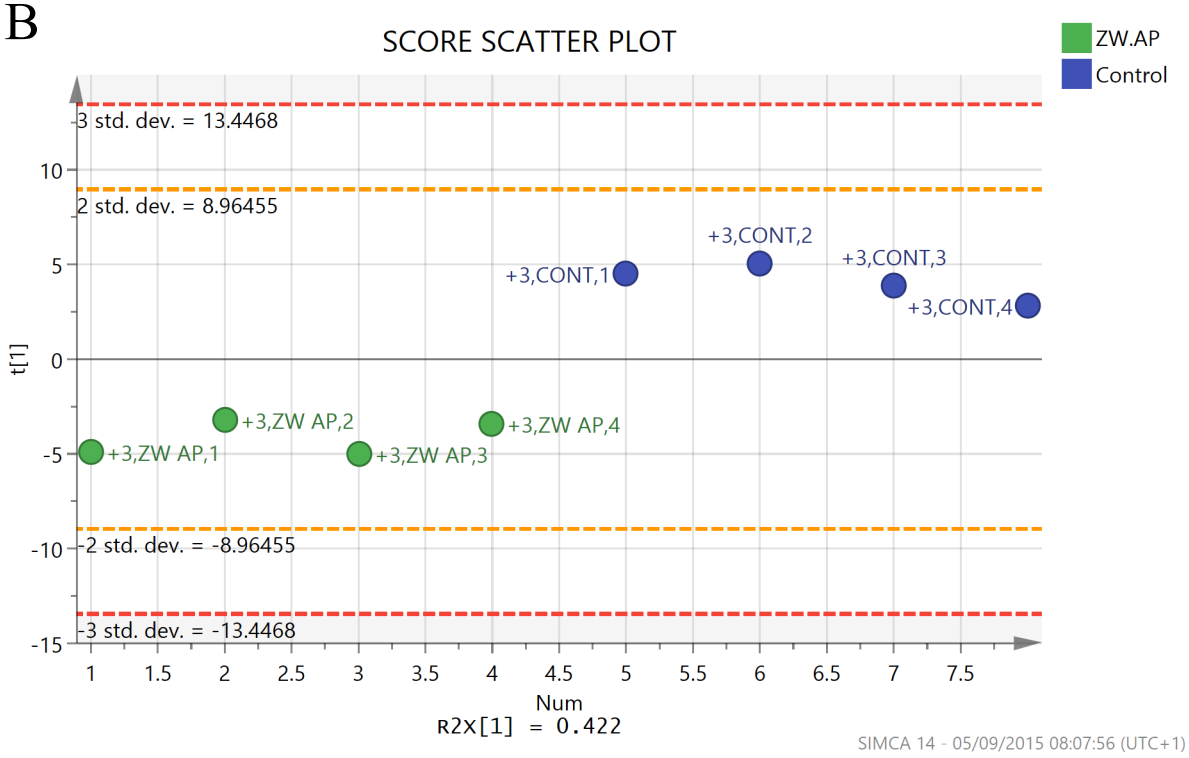
B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Control
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-3 std. dev. = -13.4468
+3,CONT,1
+3,CONT,2
+3,CONT,3
+3,CONT,4
+3,ZW AP,1
+3,ZW AP,2
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Num
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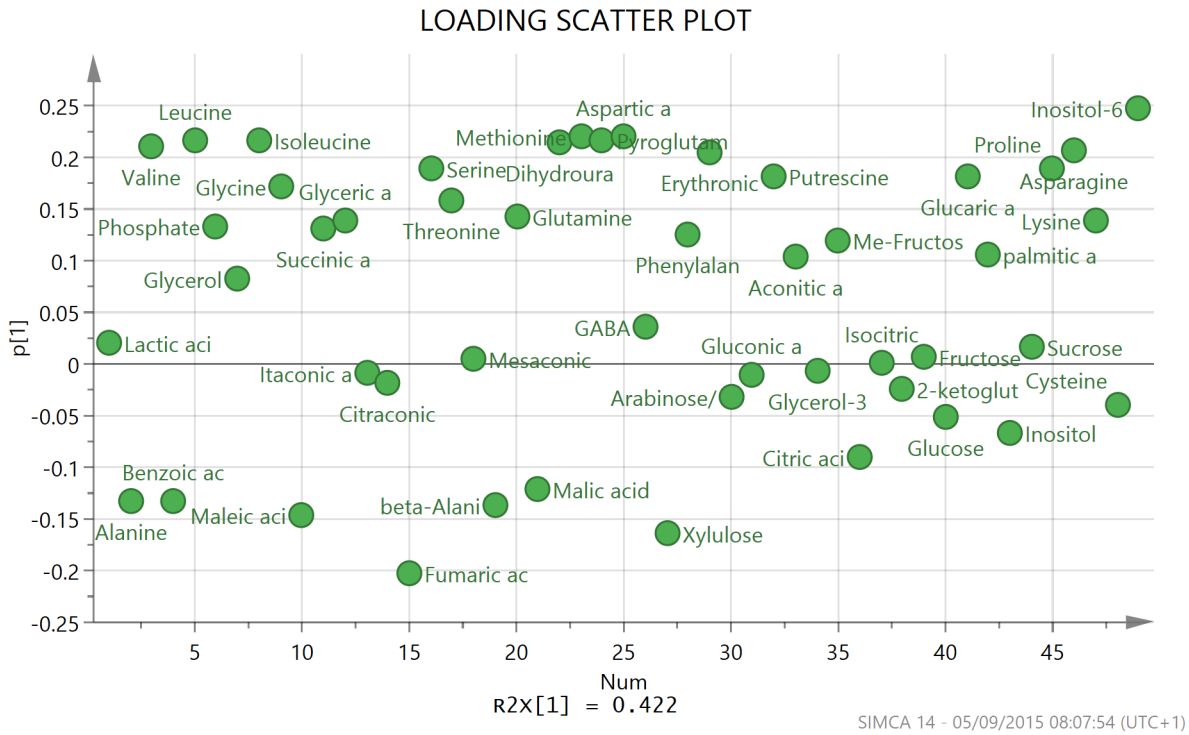
LOADING SCATTER PLOT
Leucine
Isoleucine
Valine
Glycine
Glyceric a
Phosphate
Succinic a
Glycerol
Lactic aci
Itaconic a
Citraconic
Benzoic ac
Maleic aci
Alanine
Methionine
Aspartic a
Pyroglutam
Serine
Dihydroura
Threonine
Glutamine
Fumaric ac
beta-Alani
Malic acid
Xylulose
Mesaconic
GABA
Erythronic
Putrescine
Phenylalan
Aconitic a
Gluconic a
Arabinose/
Glycerol-3
Isocitric
Citric aci
Glucose
Inositol-6
Proline
Asparagine
Glucaric a
Lysine
Me-Fructos
palmitic a
Fructose
Sucrose
2-ketoglut
Cysteine
Inositol
p[1]
Num
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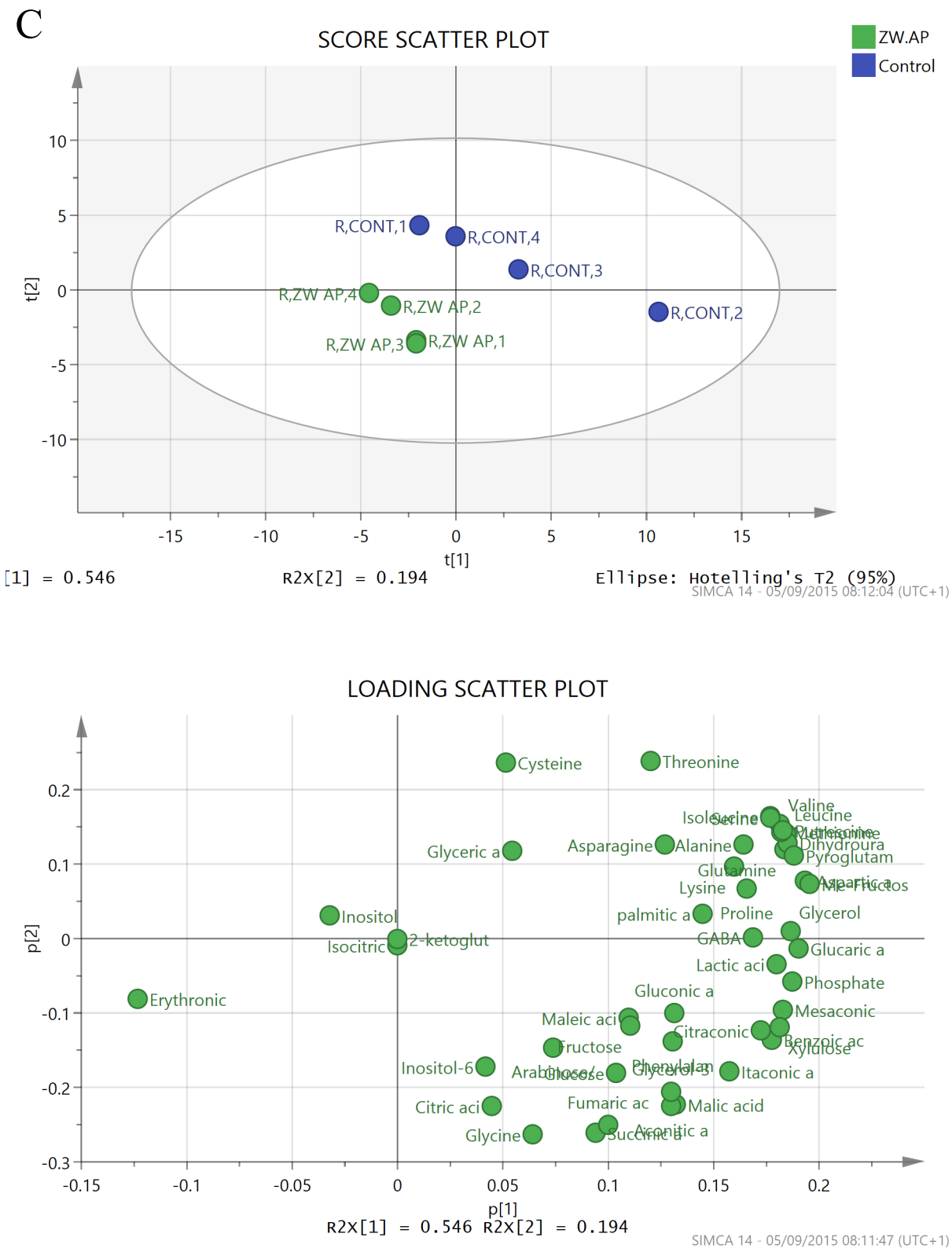
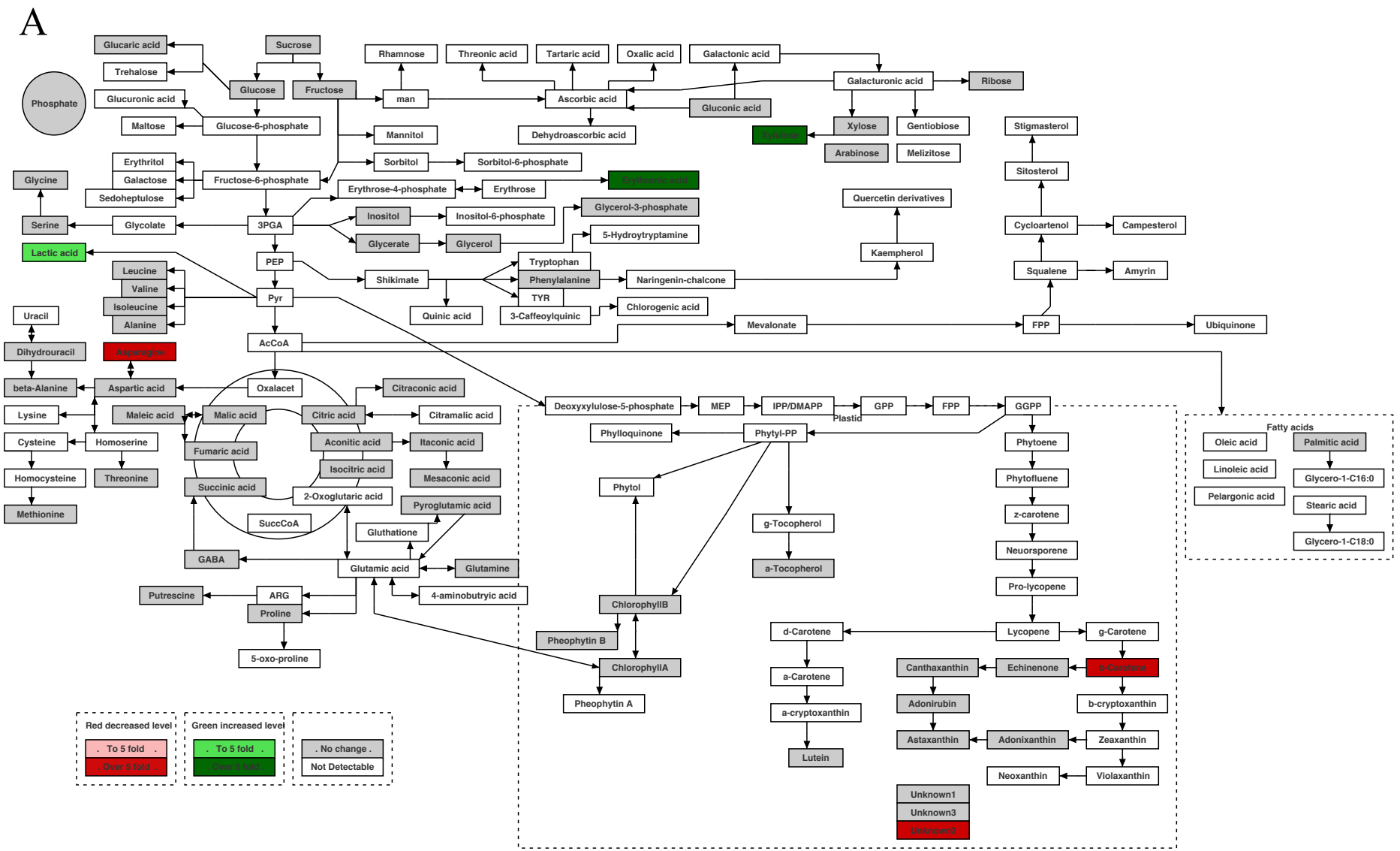



Figure 4-18 Principal component analysis (PCA) of the second generation CrtZW.APRR2 and Control tomato fruits at 3 different stages of ripening. The metabolites represented were detected by GC-MS.

Score scatter plots and loading scatter plots are showed for (A) Mature green stage, (B) 3 days post breaker and (C) ripe stages. These subsequent stages of ripening are shown as MG, +3 and R respectively in score scatter plots. The full names of the variables (metabolites) on the loading scatter

plots are available in Table 4-17. Four biological with minimum three technical replicates were analysed. The extraction method, analytical and data processing method used to analyse polar metabolites are described in sections 2.2.1.2, 2.2.1.3, 2.2.4 and 2.10

Also in CrtZW.APRR2 and its control line for a better visual comparison about the metabolite changes in fruits at each stage, the changes were illustrated in biochemical pathways (Figure 4-17). The changes are illustrated as colour change reflecting the fold changes of metabolite ratio in CrtZW.APRR2 to Control line. The visual figures help for a better understanding of the overall changes happening at different stages of ripening. For conclusion see section 4.7.2.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Sucrose
Rhamnose
Threonic acid
Tartaric acid
Oxalic acid
Galactonic acid
Trehalose
Glucose
Fructose
Galacturonic acid
Ribose
Phosphate
Glucuronic acid
man
Ascorbic acid
Gluconic acid
Maltose
Glucose-6-phosphate
Xylose
Gentiobiose
Mannitol
Dehydroascorbic acid
Arabinose
Melizitose
Stigmasterol
Erythritol
Sorbitol
Sorbitol-6-phosphate
Galactose
Fructose-6-phosphate
Sitosterol
Glycine
Sedoheptulose
Erythrose-4-phosphate
Erythrose
Quercetin derivatives
Glycerol-3-phosphate
Serine
Glycolate
3PGA
Inositol
Inositol-6-phosphate
Cycloartenol
Campesterol
Glycerate
Glycerol
5-Hydroytryptamine
Lactic acid
PEP
Tryptophan
Kaempherol
Leucine
Shikimate
Phenylalanine
Naringenin-chalcone
Squalene
Amyrin
Valine
TYR
Pyr
Isoleucine
Quinic acid
3-Caffeoylquinic
Chlorogenic acid
Uracil
Alanine
Mevalonate
FPP
Ubiquinone
Dihydrouracil
AcCoA
Asparagine
beta-Alanine
Aspartic acid
Oxalacet
Citraconic acid
Deoxyxylulose-5-phosphate
MEP
IPP/DMAPP
GPP
FPP
GGPP
Lysine
Maleic acid
Malic acid
Citric acid
Citramalic acid
Plastid
Phylloquinone
Phytyl-PP
Fatty acids
Cysteine
Homoserine
Fumaric acid
Aconitic acid
Itaconic acid
Phytoene
Oleic acid
Palmitic acid
Isocitric acid
Phytol
Phytofluene
Linoleic acid
Homocysteine
Threonine
Mesaconic acid
Succinic acid
2-Oxoglutaric acid
g-Tocopherol
z-carotene
Pelargonic acid
Glycero-1-C16:0
Methionine
Pyroglutamic acid
a-Tocopherol
Neuorsporene
Stearic acid
SuccCoA
Gluthatione
Pro-lycopene
Glycero-1-C18:0
GABA
Glutamic acid
Glutamine
Lycopene
g-Carotene
Putrescine
ARG
4-aminobutryic acid
ChlorophyllB
d-Carotene
Echinenone
b-Carotene
Proline
Pheophytin B
Canthaxanthin
5-oxo-proline
ChlorophyllA
a-Carotene
Adonirubin
b-cryptoxanthin
Pheophytin A
a-cryptoxanthin
Astaxanthin
Adonixanthin
Zeaxanthin
Lutein
Neoxanthin
Violaxanthin
Unknown1
Unknown3
Unknown2
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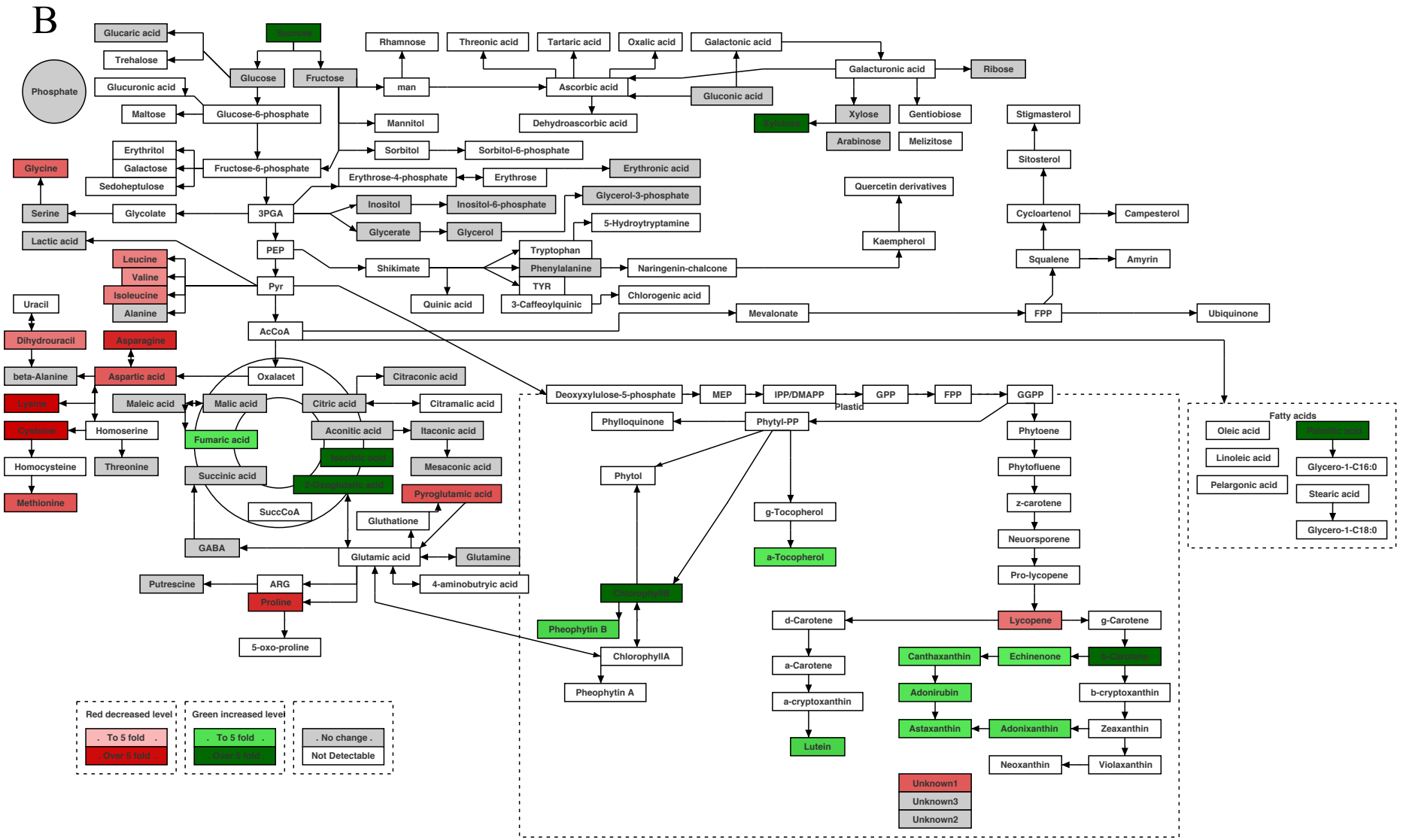
. To 5 fold .
. Over 5 fold .
Green increased level
. To 5 fold .
Over 5 fold
. No change .
Not Detectable


B
Glucaric acid
Sucrose
Trehalose
Glucose
Fructose
Rhamnose
Threonic acid
Tartaric acid
Oxalic acid
Galactonic acid
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Ribose
Phosphate
Glucuronic acid
man
Ascorbic acid
Gluconic acid
Maltose
Glucose-6-phosphate
Mannitol
Dehydroascorbic acid
Xylitol
Xylose
Gentiobiose
Arabinose
Melizitose
Stigmasterol
Erythritol
Sorbitol
Sorbitol-6-phosphate
Glycine
Galactose
Fructose-6-phosphate
Erythronic acid
Sedoheptulose
Erythrose-4-phosphate
Erythrose
Sitosterol
Quercetin derivatives
Serine
Glycolate
3PGA
Inositol
Inositol-6-phosphate
Glycerol-3-phosphate
Cycloartenol
Campesterol
Glycerate
Glycerol
5-Hydroytryptamine
Lactic acid
PEP
Tryptophan
Kaempherol
Leucine
Shikimate
Phenylalanine
Naringenin-chalcone
Squalene
Amyrin
Valine
TYR
Pyr
Isoleucine
Quinic acid
3-Caffeoylquinic
Chlorogenic acid
Uracil
Alanine
Mevalonate
FPP
Ubiquinone
Dihydrouracil
Asparagine
AcCoA
beta-Alanine
Aspartic acid
Oxalacet
Citraconic acid
Deoxyxylulose-5-phosphate
MEP
IPP/DMAPP
GPP
FPP
GGPP
Plastid
Fatty acids
Lysine
Maleic acid
Malic acid
Citric acid
Citramalic acid
Phylloquinone
Phytyl-PP
Oleic acid
Palmitic acid
Cysteine
Homoserine
Fumaric acid
Aconitic acid
Itaconic acid
Phytoene
Linoleic acid
Glycero-1-C16:0
Homocysteine
Threonine
Mesaconic acid
Phytol
Phytofluene
Pelargonic acid
Stearic acid
Methionine
Succinic acid
g-Tocopherol
z-carotene
Glycero-1-C18:0
Pyroglutamic acid
SuccCoA
Gluthatione
a-Tocopherol
Neuorsporene
GABA
Glutamic acid
Glutamine
Pro-lycopene
Putrescine
ARG
4-aminobutryic acid
Pheophytin B
d-Carotene
Lycopene
g-Carotene
Proline
ChlorophyllA
a-Carotene
Canthaxanthin
Echinenone
5-oxo-proline
Pheophytin A
a-cryptoxanthin
Adonirubin
b-cryptoxanthin
Lutein
Astaxanthin
Adonixanthin
Zeaxanthin
Neoxanthin
Violaxanthin
Unknown1
Unknown3
Unknown2
Red decreased level
To 5 fold
Over 5 fold
Green increased level
To 5 fold
Over 5 fold
No change
Not Detectable

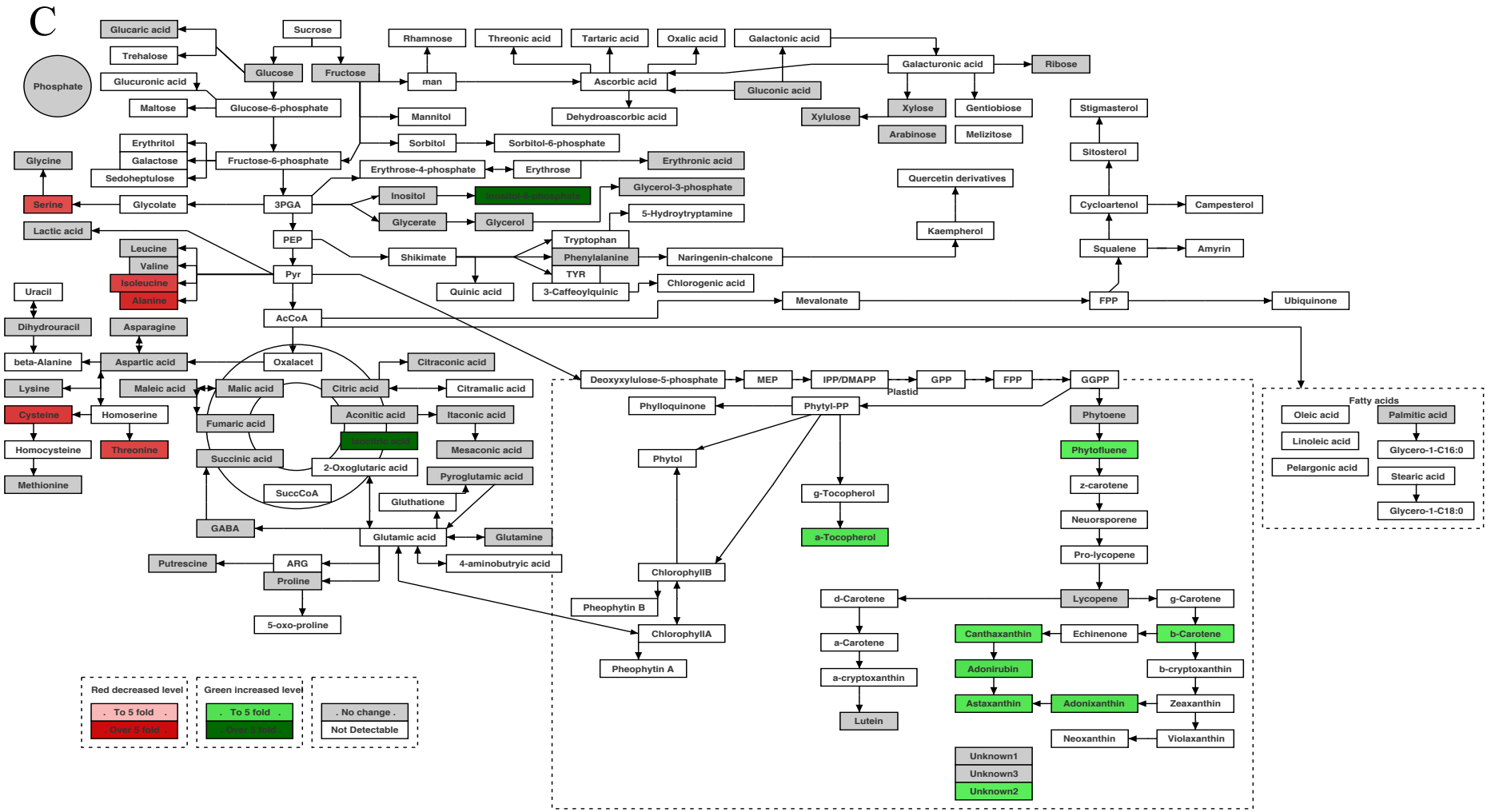



Figure 4-19 Pathway displays of metabolite changes in CrtZW.APRR2 compared Control line at different stages of fruit development and ripening

Figure 4-19 Pathway displays of metabolite changes in CrtZW.APRR2 compared Control line at different stages of fruit development and ripening

Pathway displays of metabolite changes are illustrated in (A) Mature green stage, (B) 3 days post breaker, (C) 7 days post breaker. The figures displayed metabolite changes quantitatively over schematic representations in biochemical pathway using BioSynLab software (www.biosynlab.com). The fold changes of compound levels are presented as ratio of CrtZW.APRR2 level to the Control comparator. Light red indicates up to 5 folds decrease and dark red more than 5 fold decrease whereas light green illustrates up to 5 folds increase and dark green more than 5 fold increase. Grey shows no significant changes. White indicates that the metabolite was undetectable either in the samples or in the analytical platforms used. 3PGA, glyceraldehyde-3-phosphate; Ac-CoA, acetyl-coenzyme A; ARG, arginine; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl-pyrophosphate; GPP, geranyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; SuccCoA, succinyl-coenzyme A; TYR, tyrosine.

## 4.3 Fractionation of sub-plastidial components of chloroplasts and chromoplasts in CrtZW.DET1 and the parental line of CrtZW and Control (T56.MM)

As described in section 2.4 the fractionation of sub-plastidal components of CrtZW.DET1, CrtZW and Control (T56.MM) lines were carried out at MG and ripe (3dpb to 5dpb tomato fruits) stages of ripening. As Figure 4-20 shows the sucrose density gradient consisted of 5 discontinues steps of 45%, 38%, 20% 15% and 5%. After an overnight (17hr) centrifuge the sub-plastidal compartments separated on the sucrose gradient as shown in Figure 4-20 A and B for MG and ripe stages respectively. Each experiment done in duplicate and the fractions (1ml) were collected from the top layer to the end of each tube, which normally consist of 33 fractions. Figure 4-20 C illustrate the selected fractions contained the main colour changes through gradients. All of the lines at both stages of ripening represented 2 distinct layers; while in CrtZW.DET1, CrtZW at MG stage the second layer separated in two sections. The top layer contained fraction 1 to 3 (depend on the line) and the second main layer belonged to fractions 18 to 27 which in CrtZW.DET1, CrtZW at MG stage fraction 18-22 represent the first section and fraction 23-27 belong to the second section of this layer (see Figure 4-20 A).

In sub-chromoplast fractionation experiment, a vast range of analysis consist of pigment, proteins and lipid identifications of fractions were done and published on tomato fruits (Nogueira et al., 2013). The paper conclude that fractions 1 and 2 correspond to the free plastoglobules of the chromoplasts and fractions 17 to 23 appear to be the sub compartment structure with thylakoid membrane. Fractions 24 to 28 represent thylakoid membrane plus envelope membrane structure while fractions 25 to 32 represents enriched stromal proteins (Nogueira et al., 2013; Nogueira, 2013). As the main band appeared approximately in the same fractions the same conclusion was considered for the results.

In Mature green lines, the intensity of CrtZW.DET1 was greater than CrtZW and Control lines. The light orange colour of top layer and first section of second layer, which was in due of ketocarotenoids existence at MG stage, is an important result. At ripe stage CrtZW.DET1 had the highest intensity of orange colour and CrtZW and Control line are the subsequent lines respectively.

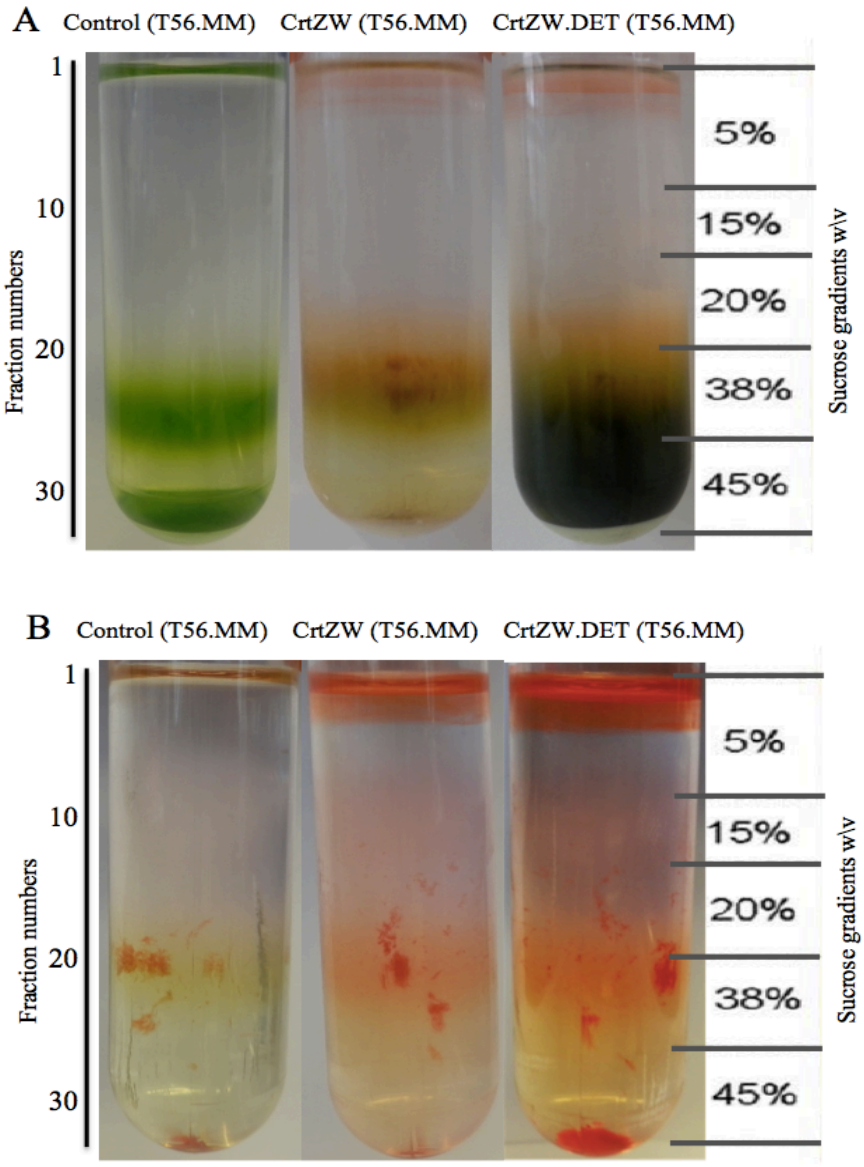

A
Control (T56.MM)
CrtZW (T56.MM)
CrtZW.DET (T56.MM)
1
10
20
30
Fraction numbers
5%
15%
20%
38%
45%
Sucrose gradients w\v
B
Control (T56.MM)
CrtZW (T56.MM)
CrtZW.DET (T56.MM)
1
10
20
30
Fraction numbers
5%
15%
20%
38%
45%
Sucrose gradients w\v
C
Control collected fractions numbers
1 2 3 10 11 15 16 17 19 20 21 23 33
CrtZW collected fractions numbers
1 2 3 11 17 19 21 24 25 28 29 33

Figure 4-20 Fractionation of sub-plastidial components of the chromoplasts of Control, CrtZW and CrtZW.DET1 lines with (T56.MM) backgrounds at MG (A) and Ripe (B) stages. (C) Selected collected fractions represent the colour changes through gradient in Control and CrtZW lines at Ripe stage.

Fractionation was performed at two steps of MG and 3-5 dpb tomato fruits as ripe samples. Chloroplasts and chromoplasts were extracted from fresh tomato fruits (80 g) and separated on two discontinuous sucrose gradient of 5%, 15%, 20%, 38% and 45% (w/v) as described in section 2.4. Fractions (1ml) were collected and stored at -80 for further analysis. A total of 33 fractions were collected per each centrifuge tube. C: represent the selected collected fractions containing the colour changes through gradients from control line and CrtZW at ripe stage for a representative instance of collected fractions.

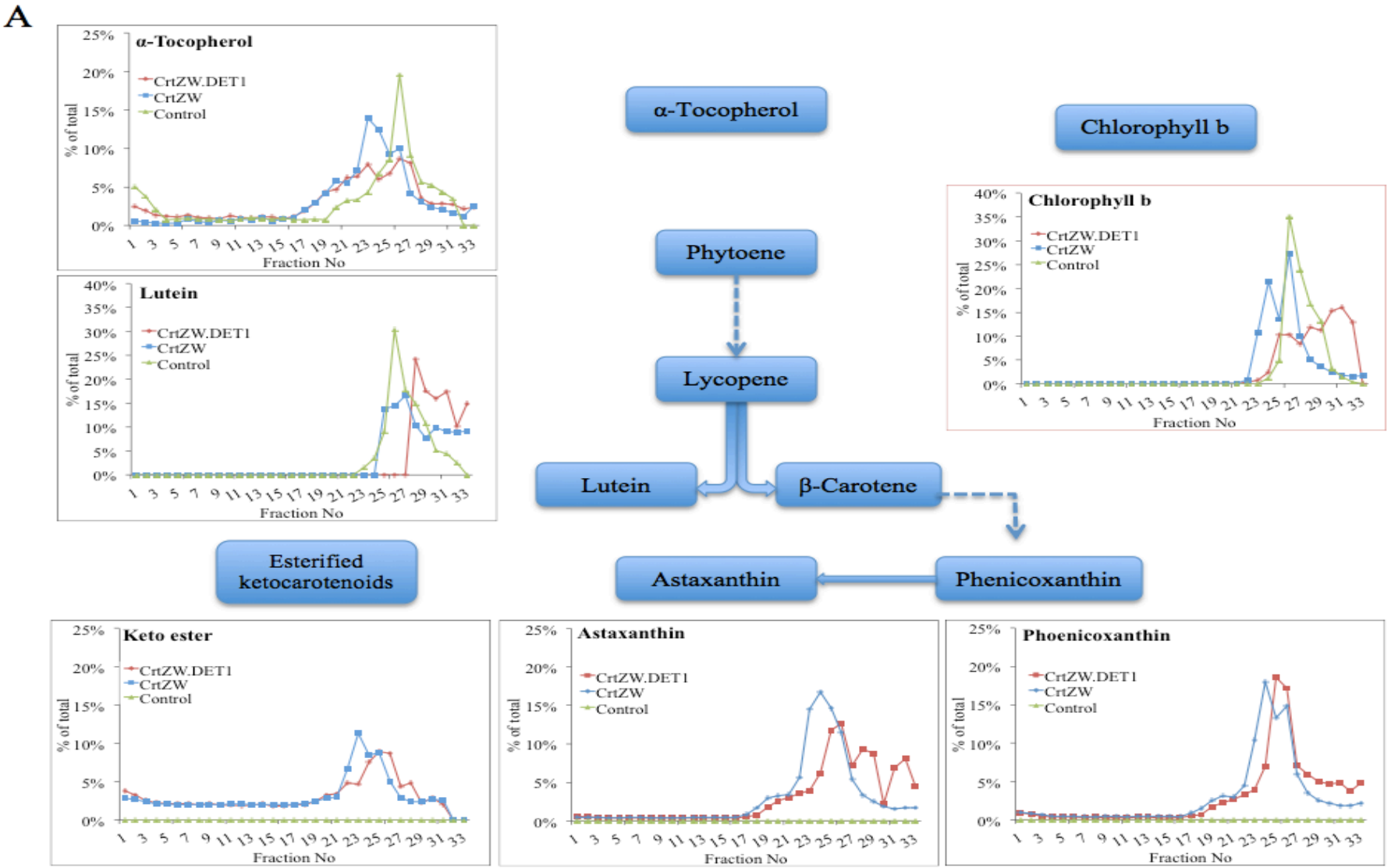
The UPLC profile of ketocarotenoids, carotenoids, chlorophylls and α-tocopherol was analysed from collected fractions of sub-chloroplast and sub-chromoplast components of CrtZW.DET1 and its derived parental and control lines at MG and Ripe stages. Distribution of pigments through gradient was analysed and the results were presented as the percentage in each fraction compared to the total content of metabolite in gradient, which represent the share of each fraction (chromoplast and chloroplast sub compartments) in storage of each pigment. The detailed pigment levels were described in section 4.2.4.1, which showed all compounds analysed were higher quantitatively in CrtZW.DET1 compared to the CrtZW parental line and also to the Control line except Lutein. Therefore, here the aim is finding out the probable differences among localization of the pigments through sub-chloroplast and sub-chromoplast component at MG and Ripe stages respectively in CrtZW, CrtZW.DET1 and Control (T56.MM) lines. In general, most carotenoids were sequestrated in the membranes (F14-29) (see Figure 4-20C). Only ketocarotenoids and α-tocopherol observed in plastoglobules (F1-3) at MG stage, explaining the light orange colour of top layer (see Figure 4-20C and Figure 4-21A). At the ripe stage phytoene and phytofluene joined to ketocarotenoids and α-tocopherol in the plastoglobules. The accumulation of metabolites in plastoglobules at ripe stage was higher to MG stage, which is in due of considerable increase in isoprenoids levels at this stage.

The pigment profile of each line had common patterns at MG and Ripe stages for some of metabolites. At the MG stage all of ketocarotenoids had a main peak in CrtZW while mostly in CrtZW.DET1 they spread through more fractions with 2-3 peaks suggesting membrane alteration. A uniform pattern of pigment profile at the ripe stage included more pigments. For example all of ketocarotenoids, α-tocopherol, phytoene, phytofluene and lycopene had one main peak for CrtZW at F22 and a

sharper peak for CrtZW.DET1 at 25, along with the availability of pigments through more fractions (F16-29 membrane components) and Control line peaked sooner than two lines of CrtZW and CrtZW.DET1 around F20 (see Figure 4-21). Higher quantity of these compounds in CrtZW.DET1 and also lower storage of metabolites in plastoglobules to CrtZW confirms that these alterations in membrane structure of CrtZW.DET1 related to its developed and expanded membranes in this line (see section 4.7.1.3 for conclusion).

To have a more detailed insight into the metabolites present in the subcomponents of chloroplast and chromoplast, each compound separation at MG and Ripe stages was examined. At MG and ripe stages α-tocopherol mainly accumulated in thylakoid and envelope membrane while at ripe stage plastoglobules also have a reasonable proportion. At MG around 2.5 and 5% of α-tocopherol stored at plastoglobules in CrtZW.DET1 and control lines respectively while at ripe stage it reached to 2, 6 and 12% for Control, CrtZW.DET1 and CrtZW respectively (Figure 4-21). Lutein at MG stage is seen mainly in stromal and envelope fractions (F23-35) with different pattern for each line but in CrtZW.DET1 it appeared with a delay just in stromal fractions (F27-33). Lutein at ripe stage accumulated at thylakoid and envelope membrane (F15-27) with different outlines in three lines. This showed lutein in different stages sequestrated in different parts (Figure 4-21). Accumulation of ketocarotenoids similar to other isoprenoids was mostly in thylakoid and envelope membranes but the plastoglobules share was also considerable. The storage of ketocarotenoids especially ketosters in plastoglobules at MG stage in CrtZW.DET1 was higher than CrtZW, which at ripe stage were vice versa. In other words the level of plastoglobular ketocarotenoids in CrtZW.DET1 remained nearly the same percentage at MG and ripe stages while in CrtZW increased up to 8% from MG to ripe (Figure 4-21). Considering the higher quantity of ketocarotenoids in CrtZW.DET1, the mentioned result conferring the fact of higher capability of its membrane at ripe stage for sequestration of these compounds. Phytofluene and lycopene, which were detectable at ripe stage, followed similar layouts but just lycopene (the most abundant carotenoid at ripe stage) observed in more fractions (F11-28) to phytofluene (F15-28) and also the interesting result of lycopene absence in plastoglobules where as the storage of phytofluene in plastoglobules detected in all the three lines of CrtZW.DET1 (4%), CrtZW (10%) and control (4%). Phytoene sequestration in plastoglobules also observed in all of the three lines (Figure 4-21).

A


α-Tocopherol
Chlorophyll b
Phytoene
Lycopene
Lutein
β-Carotene
Esterified ketocarotenoids
Astaxanthin
Phenicoxanthin
α-Tocopherol
CrtZW.DET1
CrtZW
Control
% of total
Fraction No
Lutein
Chlorophyll b
Keto ester
Astaxanthin
Phoenicoxanthin

**B**

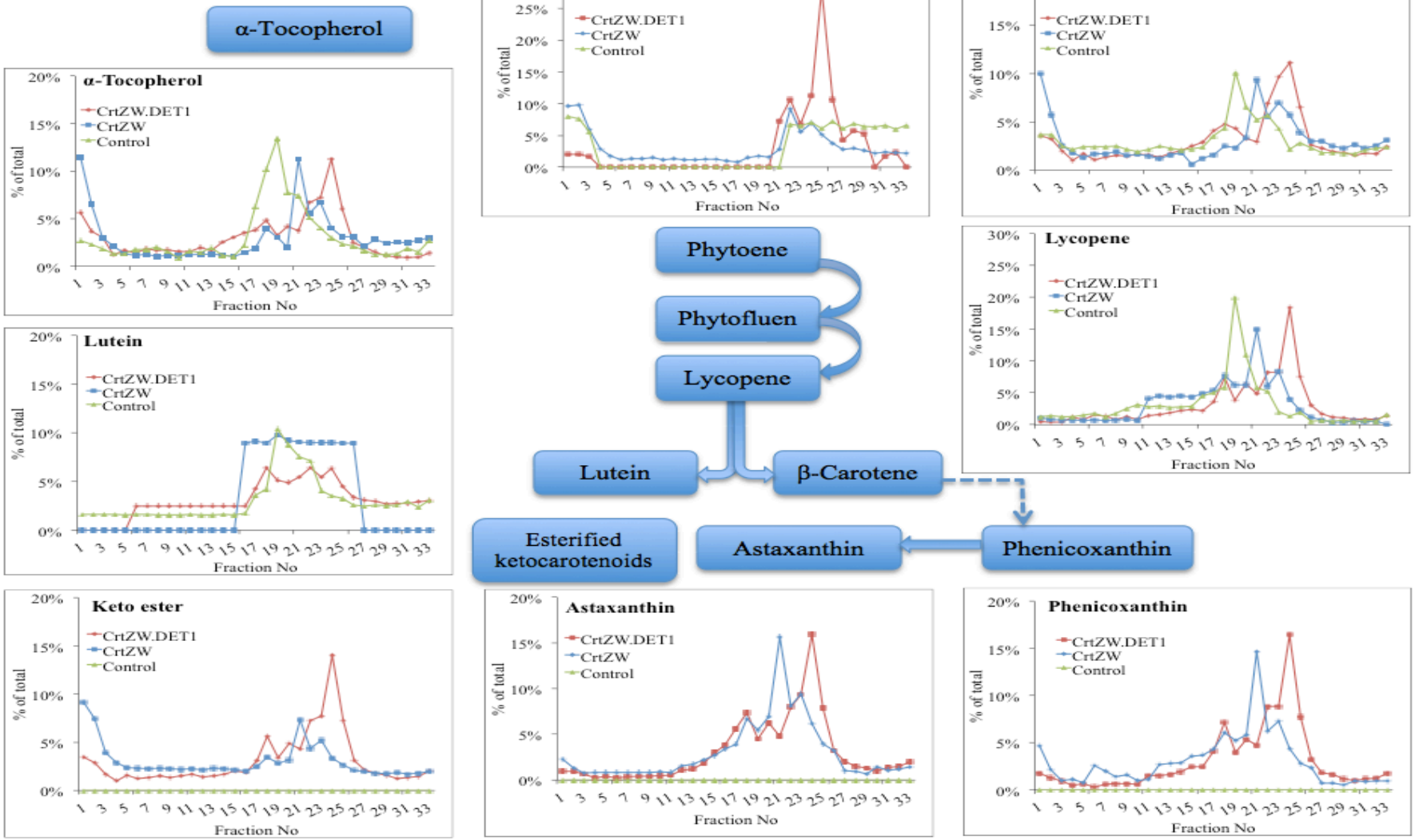

α-Tocopherol
Phytoene
Phytofluen
Lycopene
Lutein
β-Carotene
Esterified ketocarotenoids
Astaxanthin
Phenicoxanthin
α-Tocopherol
Phytoene
Phytofluene
Lycopene
Lutein
Keto ester
Astaxanthin
Phenicoxanthin
CrtZW.DET1
CrtZW
Control
% of total
Fraction No

Figure 4-21 Percentage of ketocarotenoids, carotenoids and α-tocopherol in each sub-chloroplast fraction of total content at MG stage (A) and sub-chromoplast fraction of total content at ripe stage (B).

Each graph illustrates the metabolite was extracted from 33 fractions. The summarised pathway of carotenoid biosynthesis joined with their graphs. The extraction method described in section 2.4. The results are shown as % in each fraction compared to the total content of metabolite in gradient, which is not representative for the quantity of metabolites in each fraction, but shows the share of different chromoplast and chloroplast sub compartments in storage of each pigment. The detailed pigment levels were described in section 4.2.4.1. Dashed arrows indicate existence of some intermediate steps between the two metabolites, which are not shown, to simplify the pathway. Chlorophyll b is shown at MG stage as an important pigment.

## 4.4 Transcriptional changes of some important genes

### 4.4.1 Transcriptional changes of some carotenoids biosynthesis pathway genes and DET1 gene in CrtZW.DET1 and parental and control lines.

The combination of two lines of CrtZW and DET1 by genetic crosses resulted in significant modification in metabolites and specifically in carotenoids profile (sections 4.2.4.1 and 4.2.5) of the new genotype of CrtZW.DET1. The transcriptional analyses of this line and its derived lines (CrtZW, DET1 and Control lines) have performed to ascertain how the expression patterns altered. Quantitative real-time RT-PCR was carried out at two stages of ripening 3dpb and Ripe stages as described in sections 2.3.3.2 and 2.3.3.3.

Generally, most of the changes were modest (0.5 to 2 fold) at 3 dpb except *β-lycopene cyclase gene (LCY-B)* with around 4-fold up regulation in CrtZW and CrtZW.DET1 lines to control, which was the only increase at 3 dpb. *phytoene synthase-1 (PSY-1)* were down regulated in all of the lines to control. Also *β-carotene ketolase (CrtW)* and *1-deoxy-D-xylulose-5-phosphate synthase (DXS)* expression in CrtZW.DET1 were decreased to CrtZW and control lines respectively. As expected the *DE-ETIOLATED1 (DET1)* gene showed down regulation in DET1 and CrtZW.DET1 genotypes at 3dpb (Figure 4-22).

On the whole at ripe stage most of changes were around 0.5 to 3 fold. *DXS* up regulated nearly 18 fold in DET1 line and around 6 fold in CrtZW and CrtZW.DET1 lines at Ripe stage. *PSY-1* also increased around 2 times in all of the lines in comparison to control. *β-lycopene cyclase (LYC-B)* just up regulated in CrtZW and CYC-B was the only down regulated gene at ripe stage in CrtZW and DET1 lines to Control. *DET1* gene did not show significant differences in any of the lines at ripe stage (see Figure 4-22). For conclusion about transcriptional changes see section 4.7.1.7.

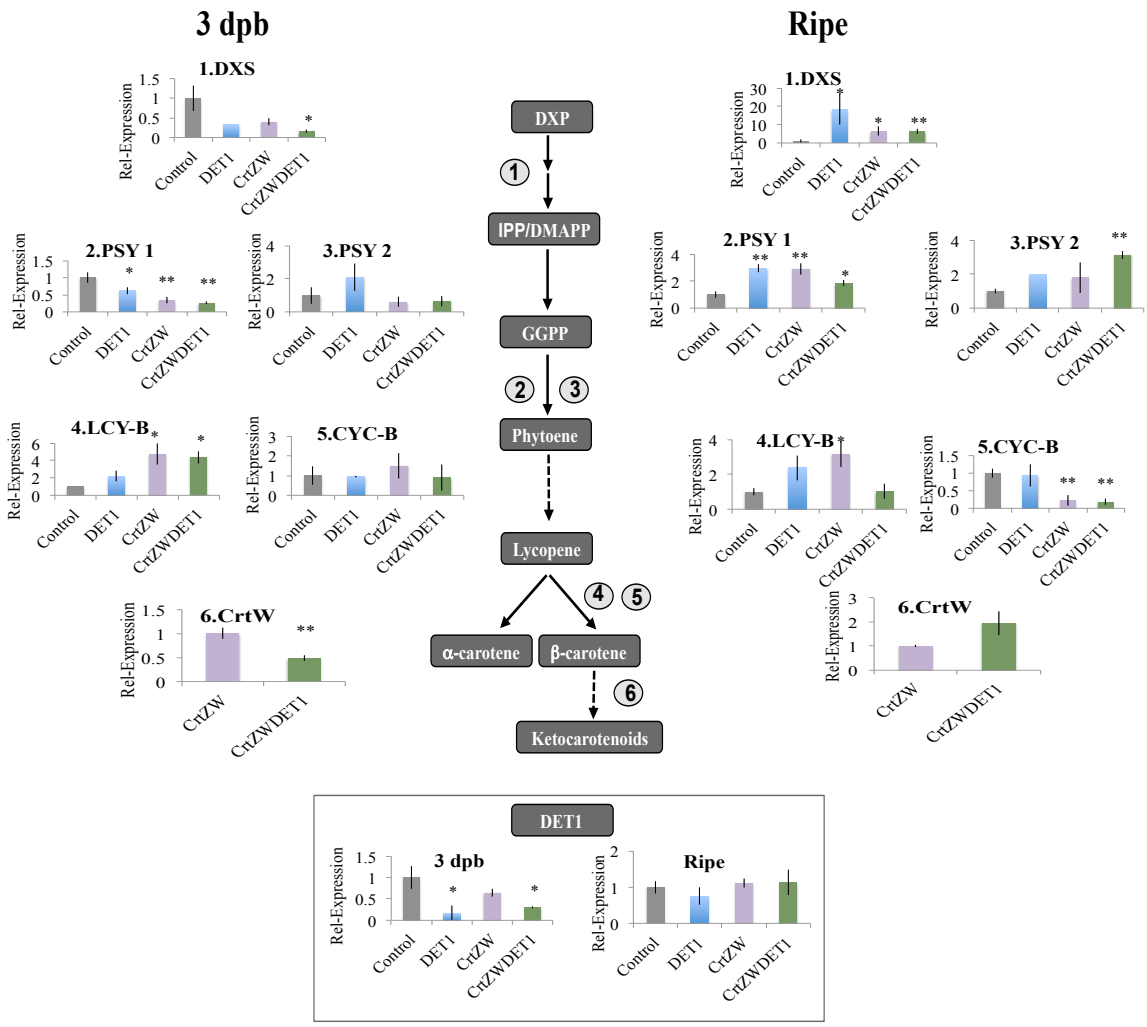



Figure 4-22 Transcript level alteration of some carotenoid biosynthesis genes and DET1 gene at 3dpb and ripe stages of ripening in CrtZW.DET1 and the derived parental lines of CrtZW and DET1 and also derived Control line.

Samples were prepared from freshly frozen fruits in liquid nitrogen as described in section 2.1.3.1. Minimum of three fruits for each of three plants for each genotype at each stage were collected and grounded in liquid nitrogen for producing a homogenous powder. RNA extraction performed as described in section 2.3.1.2. Quantitative real time RT-PCR was carried out with gene specific primers as detailed in Table 2-1. Data have normalized with the expression of actin. The result is represented as relative expression of each genotype to the expression of Control line. Error bars indicate means ± SD where n=6 to 9. For indicating statistically significant differences between control and other genotypes, Dunnett's test were performed and indicated by relative stars based on their P-values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Dashed arrows indicate existence of some intermediate steps between the two metabolites, which are not shown, to simplify the pathway. The grey bars of the histograms indicate the Control line expression level (the constant level of 1 considered for Control), which the relative expression of other lines compared to Control line. IPP, isopentenyl pyrophosphate; DMAPP, demethylallyl diphosphate; GGPP, gerenaylgeranyl diphosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; PSY-1, phytoene synthase-1; PSY-2, phytoene synthase-2; CYC-B, β-lycopene cyclase; LCY-B, β-lycopene cyclase; CrtW, β-carotene ketolase; DET1, DE-ETIOLATED1.

### 4.4.2 Transcriptional changes of some carotenoids biosynthesis pathway genes and APRR2-Like gene in CrtZW.APRR2 and parental and control lines

The combination of two lines of CrtZW and APRR2 by genetic crosses resulted in some modification in metabolites and also in carotenoids profile (see sections 4.2.4.2 and 4.2.6) of the new genotype of CrtZW.APRR2. The transcriptional analysis of this line and its derived lines (CrtZW, APRR2 and Control lines) were performed to understand how these effects performed. Quantitative real-time RT-PCR was carried out at two stages of ripening 3dpb and Ripe stages as described in section 2.3.3.2 and 2.3.3.3.

In overall, there was very little changed in gene expression in this new genotype, with a range of 0.2 to 4 fold (Figure 4-23).

At 3 dpb the only genes with modified expression pattern were *DXS* and *APRR2*. In APRR2 line *DXS* increased 3.9 fold while in CrtZW.APRR2 line nearly halved at 3 dpb. *APRR2-Like* gene up regulated in APRR2 line at both stages of ripening and in CrtZW.APRR2 genotype just at 3dpb (Figure 4-23). At Ripe stage *DXS* raised 1.7 times in APRR2 line to Control. *CrtW* gene expression decreased dramatically up to 5 fold in comparison to control, which was not expected. *CYC-B* also nearly halved in CrtZW and CrtZW.DET1 lines at this stage (Figure 4-23). For conclusion about transcriptional changes refer to 4.7.2.

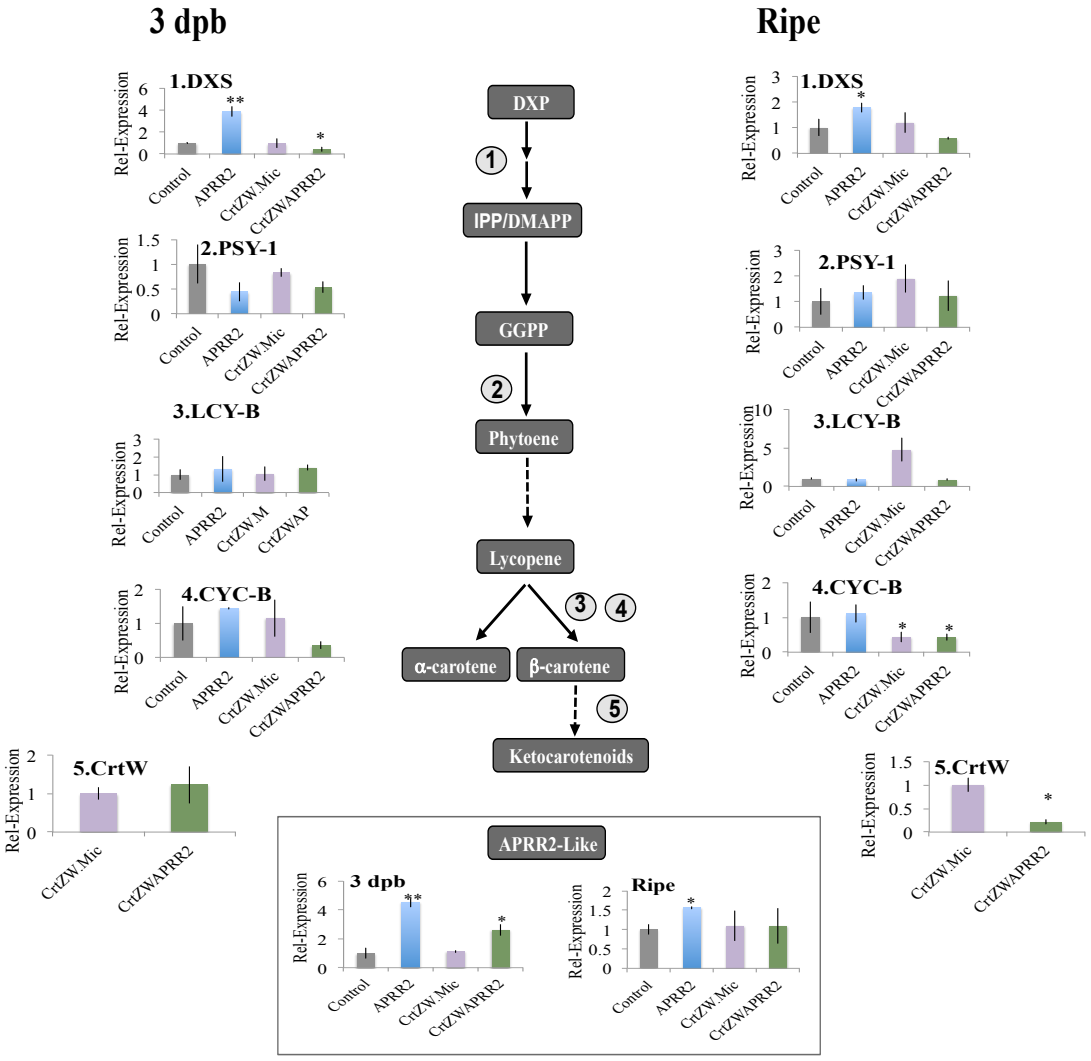



Figure 4-23 Transcript level alteration of some carotenoid biosynthesis genes and APRR2-Like gene at 3dpb and ripe stages of ripening in CrtZW.APRR2 and the derived parental lines of CrtZW and APRR2 and also derived Control line.

Samples were prepared from freshly frozen fruits in liquid nitrogen as described in section 2.1.3.1. Minimum of three fruits for each of three plants for each genotype at each stage were collected and grounded in liquid nitrogen for producing a homogenous powder. RNA extraction performed as described in section 2.3.1.2. Quantitative real time RT-PCR was carried out with gene specific primers as detailed in table Table 2-1. Data have normalized with the expression of actin. The result is represented as relative expression of each genotype to the expression of Control line. Error bars indicate means ± SD where n=6 to 9. For indicating statistically significant differences between control and other genotypes, Dunnett's test were performed and indicated by relative stars based on their P-values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Dashed arrows indicate existence of some intermediate steps between the two metabolites, which are not shown, to simplify the pathway. The grey bars of the histograms indicate the Control line expression level (the constant level of 1 considered for Control), which the relative expression of other lines compared to Control line. IPP, isopentenyl pyrophosphate; DMAPP, demethylallyl diphosphate; GGPP, gerenaylgeranyl diphosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; PSY-1, phytoene synthase-1; CYC-B, β-lycopene cyclase; LCY-B, β-lycopene cyclase; CrtW, β-carotene ketolase; APRR2-Like, Arabidopsis (Arabidopsis thaliana) pseudo response regulator2-Like gene.

## 4.5 Cellular characterisation of the CrtZW.DET1 genotypes

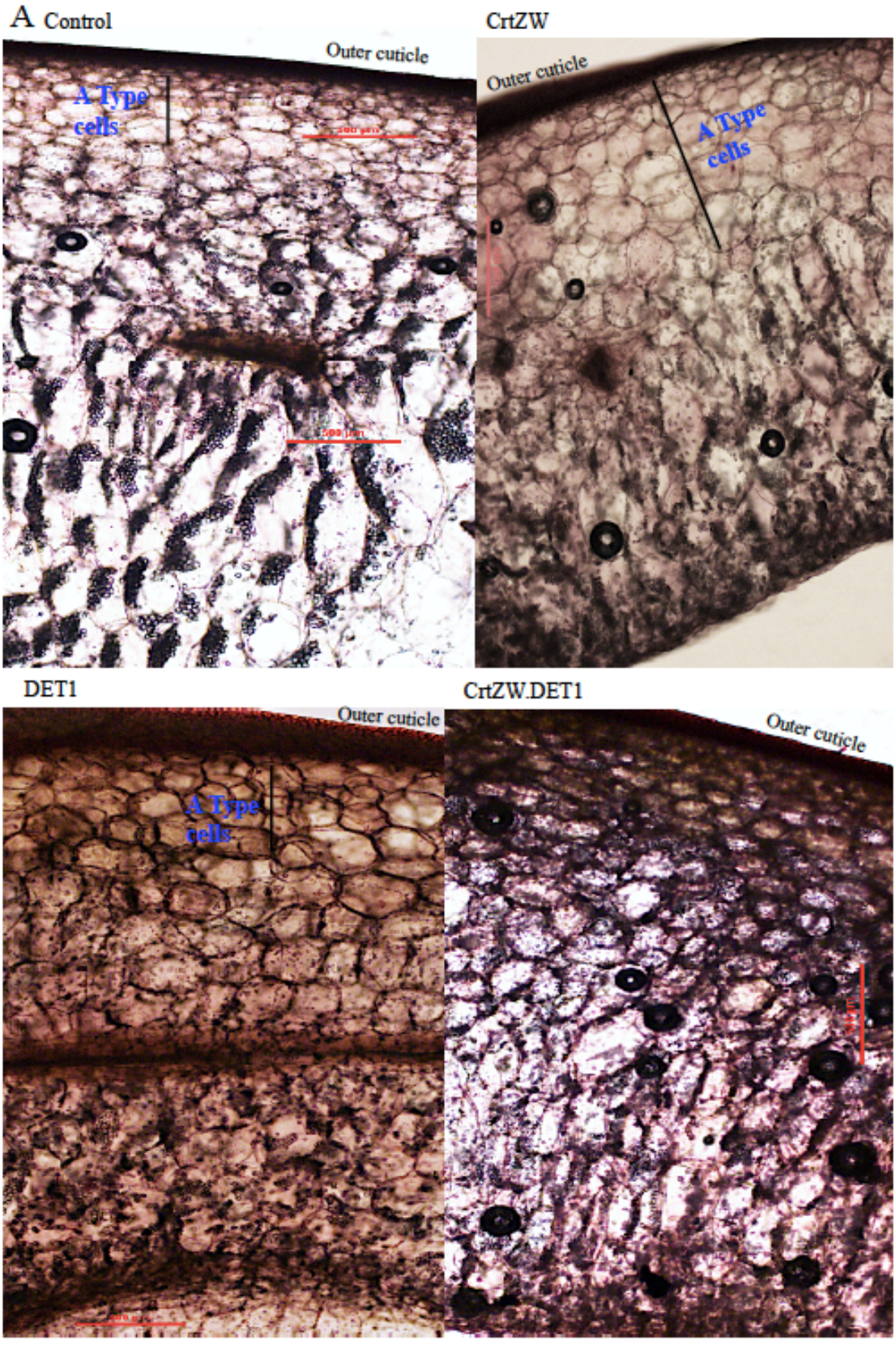
Pigment analysis of the CrtZW.DET1 lines revealed considerable changes in chlorophylls and isoprenoids levels in CrtZW.DET1 to the CrtZW and also control lines (Section 4.2.4.1).

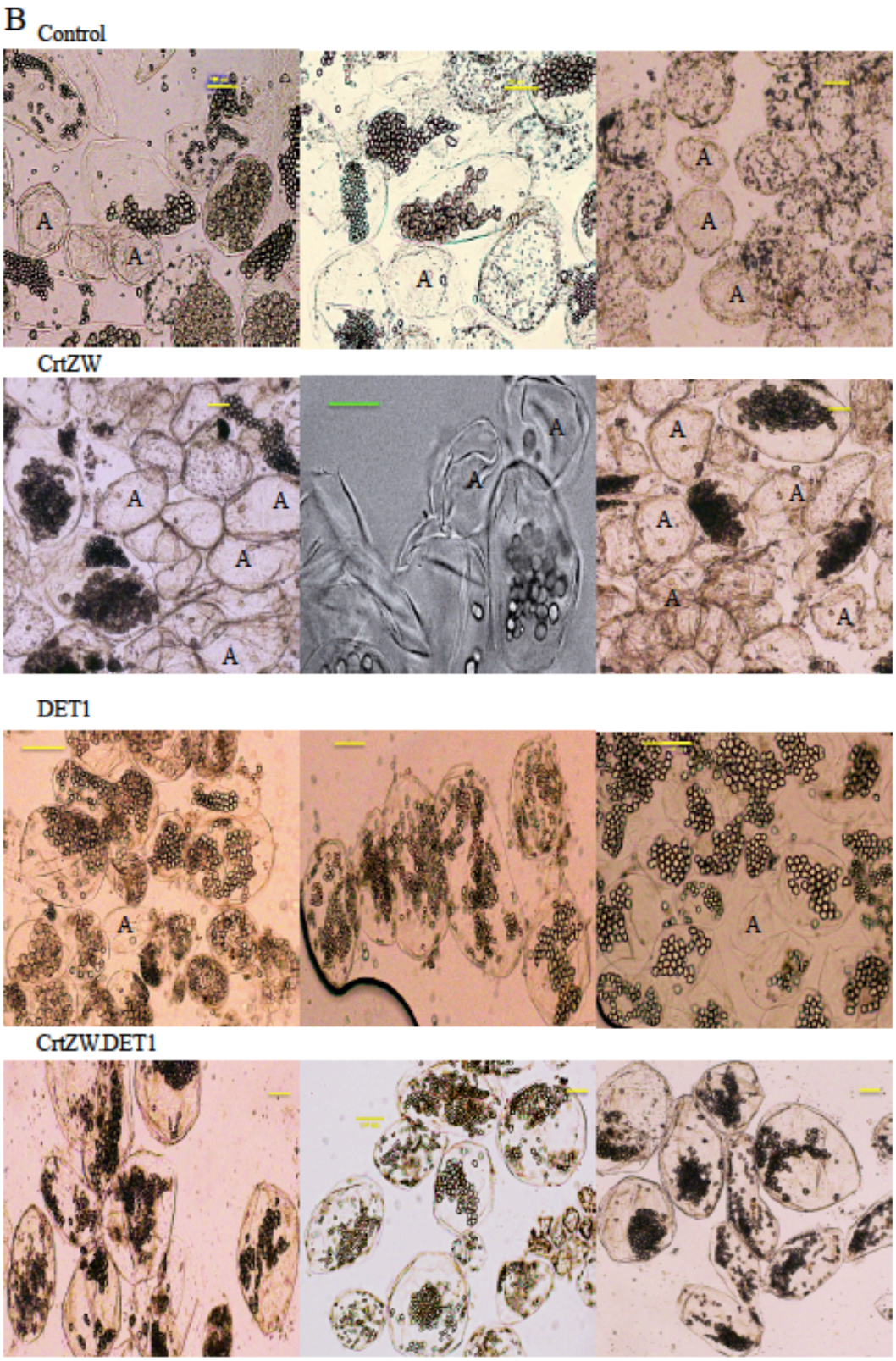
From the fractionation of plastid structures performed at the MG and ripe stages higher pigment levels (pigment analysis data) were found in plastid membranes. Alteration and expansions in plastidal membranes concluded from fractionation data at MG and ripe stages (Section 4.3). For a more comprehensive analysis of the changes in cellular and plastidal structures microscopy was performed on these lines completing the biochemical analysis. Whole pericarp slides and cell slide were prepared for investigation as described in Section 2.6.1.2 and 2.6.1.1 respectively.

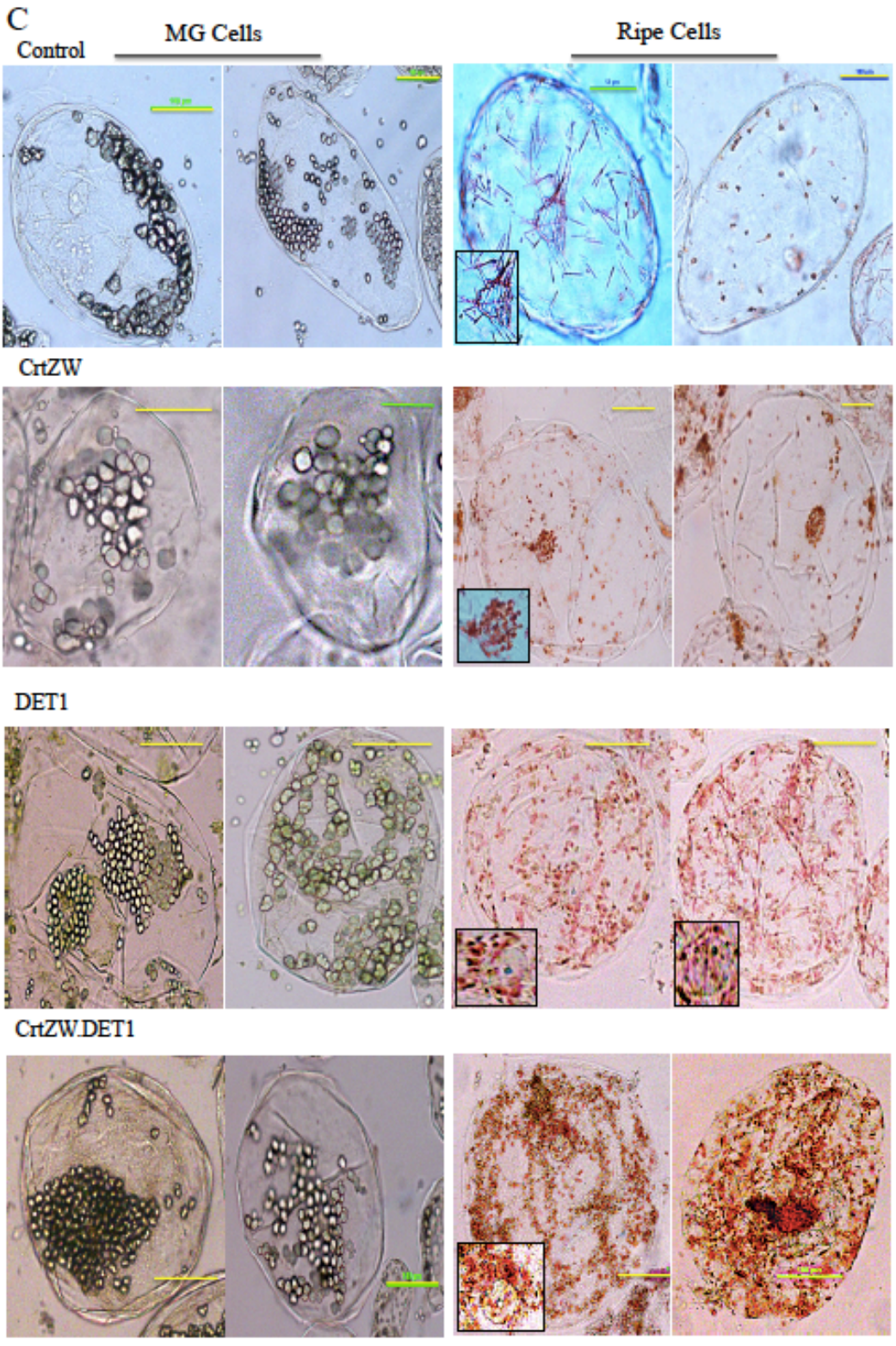
Firstly for an overview about the cell types of pericarp of these 4 lines, pericarp sections have investigated under Nomarski microscope (Figure 4-24 A). In the outer pericarp of CrtZW, DET1 and Control lines, some pericarp cells with no or very low chloroplast numbers were observed which in Figure 4-24 part A and B named as A type cells. In CrtZW nearly half of pericarp cells were A type of cells. Control and DET1 were the second and third lines containing A type cell respectively. In CrtZW.DET1 line rarely cells with no or low chloroplasts were observed and even very young cells contained considerable numbers of small chloroplasts (Figure 4-24 B).

As explained, some of outer pericarp cells contained low numbers of chloroplasts, therefore individual cells from inner pericarp were chosen from cell slides for plastid phenotypes characterisation at MG and Ripe stages. As the first impression at MG stage, the colour intensity and chloroplast numbers of CrtZW.DET1 and DET1 were observed higher than CrtZW and Control lines (Figure 4-24 B MG Cells). The colour intensity of CrtZW at the ripe stage was the lowest intensity among other lines (CrtZW.DET1, DET1 and control). At Ripe stage the chromoplast numbers and colour intensity of CrtZW.DET1 and DET1 were the first and the second highest ones respectively. The aggregation of pigments both in the form of needle-like crystalized chromoplasts (small numbers in CrtZW and rarely observed in CrtZW.DET1) and round-shape chromoplasts observed specifically around the nucleus in all of the lines (Figure 4-24 C Ripe Cells). Florescence microscopy was used for detection of nucleus position within the cell and also for better emplacement and finding the location of

chromoplasts where needed (Figure 4-24 D). Another interesting observation was seldom detection of needle-like crystals form chromoplasts in CrtZW.DET1 Ripe cells despite of the highest colour intensity and large chromoplast numbers of this line compared to other lines. See sections 4.7.1.1 and 4.7.1.2 for conclusion.


A Control
Outer cuticle
A Type cells
CrtZW
Outer cuticle
A Type cells
DET1
Outer cuticle
A Type cells
CrtZW.DET1
Outer cuticle


B
Control
CrtZW
DET1
CrtZW.DET1


C
MG Cells
Ripe Cells
Control
CrtZW
DET1
CrtZW.DET1

D

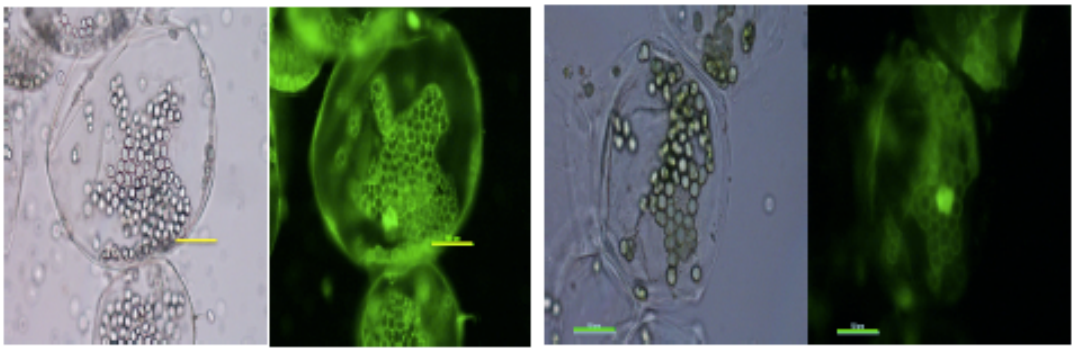


Figure 4-24 Nomarski differential interference and fluorescence microscopy images of CrtZW.DET1 and it's derived parental and control cells and plastid phenotypes at MG and Ripe stages of ripening.

(A) Pericarp section images for overall comparison of cell types in 4 lines of CrtZW.DET1, CrtZW, DET1 and Control. A type cells represents cells with low content or no chloroplasts that observed in three lines of CrtZW, DET1 and Control. Pericarp sections of MG fruits (100-200 µm) were prepared as described in section 2.6.1.2 and observed under Nomarski Microscopy. Red scale bars represent 500 µm. (B) Representative images of separated cell types in the 4 named lines at MG stage. A letters represents cells with low content or no chloroplasts that named in three lines of CrtZW, DET1 and Control. Yellow scale bars indicate 100 µm and green scale bars show 50 µm. (C) Plastid characterization of the 4 named lines at MG and Ripe stages of ripening. Aggregation of chromoplasts and crystals around nucleus also showed as zoomed pictures at ripe pictures. Samples were prepared as described in section 2.6.1.1. (D) Florescence Microscopy also used for determination of nucleus position within the cells and also for better characterization of chloroplasts where needed. Yellow scale bars indicate 100 µm and green scale bars show 50 µm.

Quantitative analysis of cellular structures and subsequent statistical analysis of cell area and chloroplast area and numbers were performed for a more precise cellular and plastidal characterisation of these lines. Two lines of CrtZW.DET1 and Control lines were focused for these assessments. Three biological replicates with 8 to 12 different measurements through various parts of pericarp from CrtZW.DET1 and control lines were measured for chloroplast area, number and cell area quantifications. As Table 4-18 indicates, chloroplast numbers of CrtZW.DET1 were nearly twice that of the Control lines. With the large variation observed in the cell area and chloroplast area of these two lines, no significant differences were observed between them. Chloroplast area to cell area ratio did not show any changes in any of the lines (Table 4-18). For an overall insight to the parental lines of CrtZW and DET1, cell and chromoplast area, chloroplast numbers were measured in one biological replicate with 8 different samples of different parts of pericarp. CrtZW represent smaller cell area, chloroplast area and also lower cell numbers. But the only significant change in DET1 was higher numbers of chloroplasts (Table 4-18). These primary data need to be confirmed by more robust data.

In conclusion most of CrtZW.DET1 cells compared to Control line at MG stage contained higher numbers of chloroplasts with more intensity (Lack of A type cells) in microscopy analysis; non A type cells had higher numbers of chloroplasts average and also higher intensity of chloroplasts in comparison to A type cells. Considerably higher numbers of chromoplasts with higher colour intensity observed in CrtZW.DET1 line to other lines at ripe stage as well. This result proposed that in CrtZW.DET1 line not only the level of pigments increased within cells but also some structural pericarp cells alteration also happened in lines with downregulated *DET1* gene which explained in section 4.7.1.1.

Table 4-18 Chloroplast area and number, cell area and cellular to chloroplastal area ratio of CrtZW.DET1 and its parental and control lines.

Cell slides were prepared as described in section 2.6.1.1 and used for detection and measurements of above parameters in the 4 lines of CrtZW.DET1, CrtZW, DET1 and Control. All cells and chloroplast areas and chloroplast numbers per cell were measured using NIS Elements version 4.2 software. All measurements done for at least three biological replicate with 8-12 measurement trough different parts of pericarp for each biological replicate. (^) In CrtZW and DET1 lines all of data were measured in one biological replicate with 8 different samples from different parts of pericarp; confirmation of these results needs more robust data.

| | Cell area average (µm2) | Chloroplast area average (µm2) | Number of Chloroplast average | Chloroplast area/cell area |
|---|---|---|---|---|
| Control | 118711 ± 51807 | 31588 ± 18305 | 62±14 | 0.27 ± 0.09 |
| CrtZW^ | 27231 ± 15431*** | 7208 ± 5324*** | 36±20** | 0.24 ± 0.1 |
| DET1^ | 122723 ± 49989 | 24355 ± 5510 | 113 ± 41* | 0.23 ± 0.1 |
| CrtZW.DET1 | 142039 ± 91967 | 39768 ± 24995 | 123 ±48*** | 0.29 ± 0.04 |

## 4.6 Cellular and plastidal characterization of CrtZW.APRR2, the parental and control lines

Pigment analysis of CrtZW. APRR2 and its derived lines represented some changes in chlorophylls and isoprenoids levels in CrtZW.APRR2 to the CtrZW.Mic and also control lines (section 4.2.4.2). For a better understanding about cellular and plastidal level of these changes, microscopy analysis was performed as a complementary method for these lines. Whole pericarp slides and cell slide were prepared and studied as described in section 2.6.1.2 and 2.6.1.1 respectively.

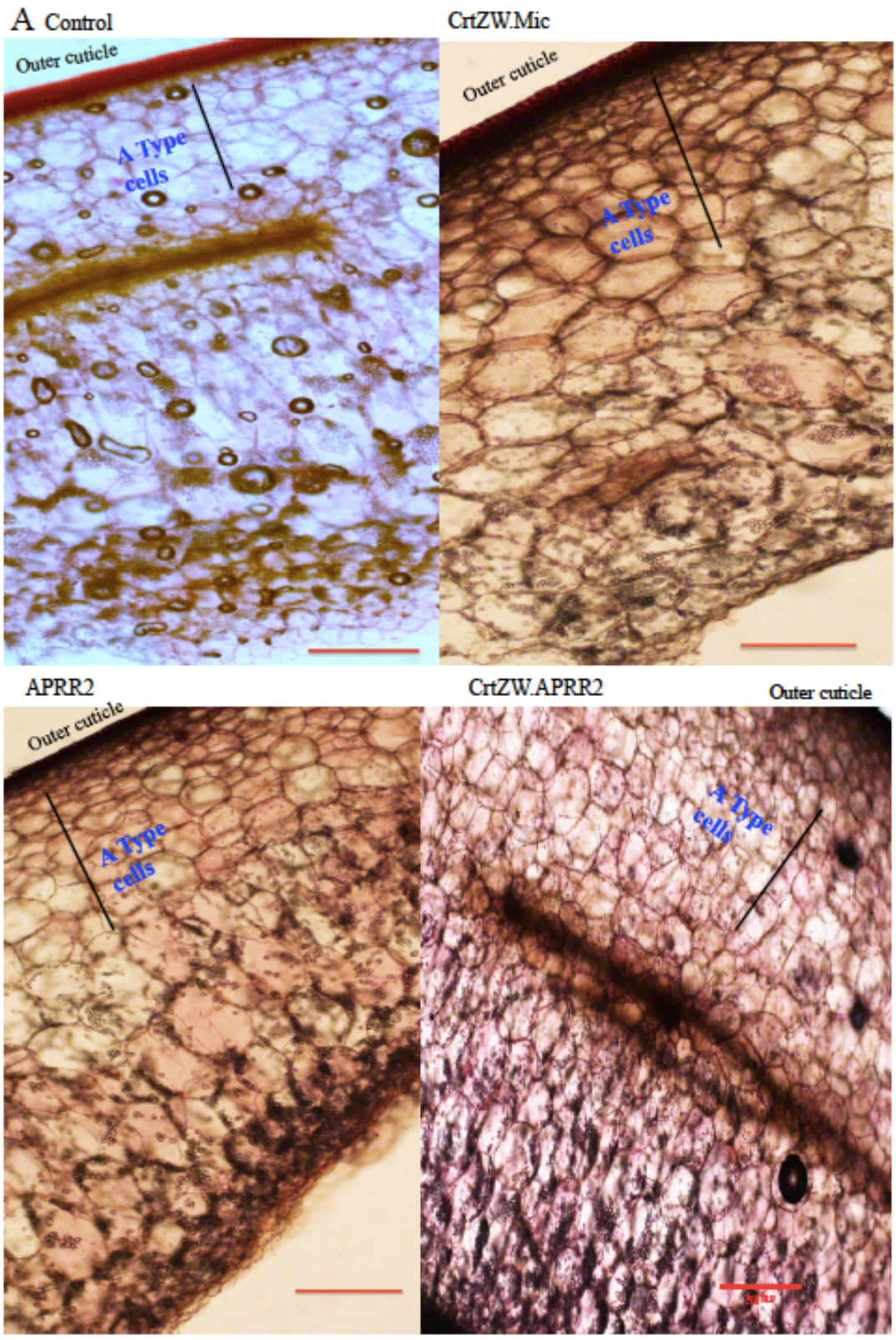
For characterisation of pericarp cell types of these 4 lines, pericarp sections have observed under Nomarski microscope (Figure 4-25 A). In the outer pericarp of all lines, some pericarp cells with no or very low content of chloroplast numbers were

observed which in Figure 4-25 part A named as A type cells. In all of the four lines, A type cells observed clearly (Figure 4-25 A).

As reported above, many of outer pericarp cells contained low numbers of chloroplasts, therefore individual cells from inner pericarp were selected from cell slides for plastid phenotypes characterization at MG and also at ripe stage.

At MG stage, the colour intensity of CrtZW.APRR2 and APRR2 were higher than CrtZW.Mic and Control lines (Figure 4-25 B MG Cells).

At ripe stage the chromoplast numbers of CrtZW.APRR2 were observed higher than other 3 lines. Both form of needle-like crystalized chromoplasts (very small amounts in CrtZW.Mic) and round shaped chromoplasts observed in all of the 4 lines (Figure 4-25 B Ripe Cells).


A Control
Outer cuticle
A Type cells
CrtZW.Mic
Outer cuticle
A Type cells
APRR2
Outer cuticle
A Type cells
CrtZW.APRR2
Outer cuticle
A Type cells

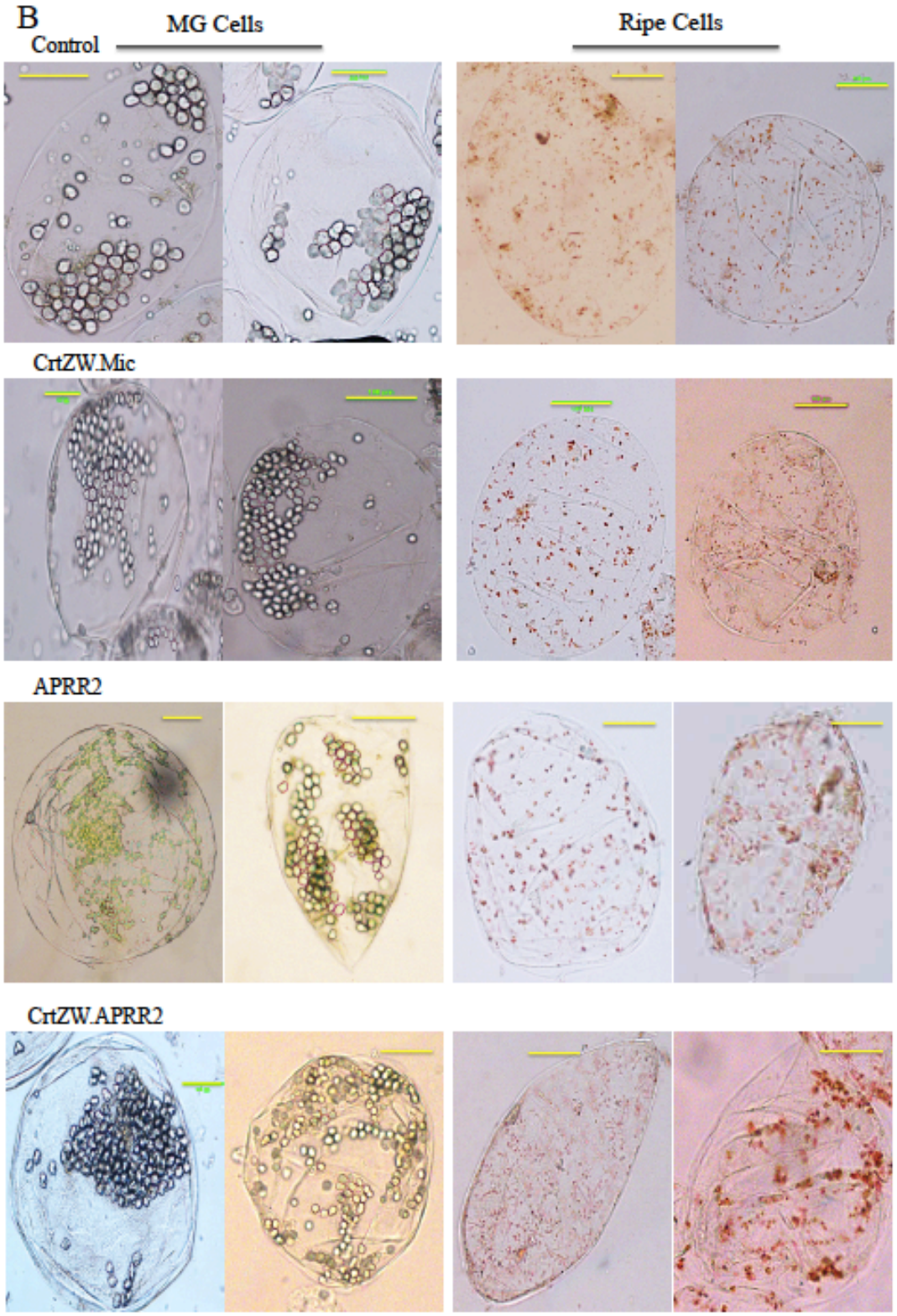



Figure 4-25 Nomarski differential interference microscopy images of CrtZW.APRR2 and its derived parental and control plastid phenotypes at MG and Ripe stages of ripening.

(A) Pericarp section images for overall comparison of cell types in 4 lines of CrtZW.APRR2, CrtZW.Mic, APRR2 and Control. A type cells represents cells with low content or no chloroplasts that observed in all of the lines. Pericarp sections of MG fruits (100-200 μm) were prepared as described in section 2.6.1.2 and observed under Nomarski Microscopy. Red scale bars represent 500 μm. (B) Plastid characterization of the 4 lines of CrtZW.APRR2, CrtZW.Mic, APRR2 and Control at MG and Ripe stages of ripening. Samples were prepared as described in section 2.6.1.1. Yellow scale bars indicate 100 μm.

As the changes in carotenoids and other metabolites were not very impressive in CrtZW.APRR2 line, just cellular and chloroplast indexes quantified in CrtZW.APRR2 and Control lines. Three biological replicate with 8 to 12 different measurements through various parts of pericarp from CrtZW.APRR2 and control lines were measured for chloroplast area, number and cell area quantifications. The chloroplast numbers of CrtZW.APRR2 did not show any difference with Control line. The cell area and chloroplast area of CrtZW.APRR2 line were around two times smaller than Control line. Chloroplast area to cell area ratio did not show any changes.

Collectively at the MG stage higher colour intensity of chloroplasts observed in CrtZW.APRR2 and APRR2 lines while at ripe stage higher numbers of chromoplasts observed for CrtZW.APRR2 line. Decrease in cell size and also chloroplast area of CrtZW.APRR2 to control line at MG stage were another significant changes of this line in microscopy analysis. For cell composition type, significant changes did not observed between the 4 lines (nearly the same sections of A type cells observed in all of the lines). Higher numbers of chromoplasts observed in CrtZW.APRR2 to other lines at ripe stage.

Table 4-19 Chloroplast area and number, cell area and cellular to chloroplastal area ratio of CrtZW.APRR2 and control lines.

Cell slides were prepared as described in section 2.6.1.1 and used for detection and measurements of above indexes in the 4 lines of CrtZW.APRR2 and Control. All cells and chloroplast areas and chloroplast numbers per cell were measured using NIS Elements version 4.2 software. All measurements done for at least three biological replicate with 12 measurements through different parts of fruit for each biological replicate.

| | Cell area average (μm2) | Chloroplast area average (μm2) | Number of Chloroplast average | Chloroplast area /cell area |
|---|---|---|---|---|
| Control | 119140 ± 48954 | 41500 ± 21954 | 79 ± 24 | 0.36 ± 0.14 |
| CrtZW.APRR2 | 62835 ± 37689 ** | 17749 ± 10412 ** | 70 ± 25 | 0.3 ± 0.11 |

## 4.7 Discussion

The genetic manipulation of astaxanthin and other ketocarotenoids as high-value secondary metabolites have been targeted for many years. As a result the production of ketocarotenoids in a variety of plants (Arabidopsis, tomato, carrot, tobacco, potato and etc.) with different quantities has occurred (Stålberg et al., 2003; Ralley et al., 2004; Jayaraj et al., 2008; Hasunuma et al., 2008; Gerjets and Sandmann, 2006). The strategy used in this study for improving the production of ketocarotenoids was using the advantage of regulatory genes (*DET1*) and transcription factors (*APRR2*) for enhancing the precursor pool of primary metabolites. Using the method of genetic crosses for the production of new genotypes is a routine method that can be used in other studies. The genetically crossed new genotypes of CrtZW.DET1 and CrtZW.APRR2 resulted in improved ketocarotenoids content.

### 4.7.1 Multi-level effects of DET1 gene modulation for the improvement of ketocarotenoids and other metabolites

#### 4.7.1.1 Not only higher metabolites but also more organelles and cells

As shown previously there is some connection between changes in carotenoid composition with plastid biogenesis and morphology (Park et al., 2002). Chromoplast sequestrated carotenoids in lipoprotein structures (Vishnevetsky et al., 1999; Bartley and Scolnik, 1995) and chromoplasts act as metabolite sink to control carotenoid accumulation, these findings show the importance of plastid differentiations and its key role in carotenoid accumulation in plants (Lu et al., 2006; Li and Van Eck, 2007). The microscopy results on CrtZW.DET1 compared to its derived lines confer that the noticeable increase of ketocarotenoids was not only a metabolite increase within cells, but also subsequently an increase in the number of plastids at subcellular organelles and finally an overall growth in the number of cells containing more plastids at the cellular levels in different stages of ripening.

The metabolite increases in CrtZW.DET1 line was in result of down regulated *DET1* gene. Down regulated *DET1* affects the light signal transduction pathways mainly affect on photosynthesis and therefore more fixation of $CO_2$ to sugars (Enfissi et al., 2010). This core metabolic changes result in plastids biogenesis, providing appropriate cell compartmentalisation for these processes, which increase in plastid area resulted in elevated plastid numbers (Enfissi et al., 2010). The alterations at

chloroplast level in ZW.DET1 line just observed in increased number of chloroplasts but not their area per cell. This could be the effect of *CrtZW* gene. In higher plants ABA is produced from the carotenoid pathway with β-carotene the precursor for downstream xanthophylls like zeaxanthin and violaxanthin (Tan et al., 1997; Burbidge et al., 1999). The considerable reduction in the level of β-carotene in *CrtZW* gene containing lines proposed lack of enough precursors for ABA production on time which result delay in ripening (this aspect of CrtZW.DET1 line with some complementary experiments will be explained fully in the next chapter as a separate chapter). Delays in ABA production could be responsible for the observed cell deficiencies as it is demonstrated that ABA has some roles not only in ripening but also in plant growth and development (Zhang et al., 2009; Galpaz et al., 2008). The *hp3* (high pigment tomato mutant with deficiency in ABA production) represent cell deficiency and fruit weight loss by 50% of total weight (Zhang et al., 2009; Galpaz et al., 2008). The overall look on CrtZW line indicated lower number of chloroplast and also decreased chloroplast area. The explained fact could justify the decrease in cell size and number in CrtZW line in result of delayed in ABA production. It can be concluded that the effect of *CrtZW* gene in ZW.DET1 line limited the effect of *DET1* gene to elevation of chloroplast numbers but not chloroplast area.

The approximately equal quantity of chlorophylls and ketocarotenoids in CrtZW line (see section 4.2.4.1 and Table 4-10) could be responsible for decreased intensity of chloroplasts under microscope, as the existence of orange colour of ketocarotenoids at from MG stage faded the sharp green colour of chloroplasts while quantification of chlorophyll level did not show significant change to control. The same observation was true for CrtZW.DET1 line that the intensity of their chloroplasts was slightly lower to DET1 line (the intensity to control line was much higher) but the chlorophylls content was not different. This means in transgenic lines producing ketocarotenoids from the MG stage need complete pigment analysis in addition to microscopy to be judged their chlorophyll content. It is also important to have an overall cellular assessment about the type of cells in in outer and inner pericarp, as the increased number of cells containing chloroplasts in outer pericarp of CrtZW.DET1 line probably had an important role in increasing the level of metabolites (lower number of A type cells). The inner and outer pericarp have shown fundamental differences within them (Montgomery et al., 1993). In this study only in the outer pericarp, A type cells (cells with no or very low chloroplast numbers) have observed.

In the three derived lines of CrtZW, Control and DET1 A type cells were more frequent (CrtZW>Control>DET1) and the morphological differences between outer and inner pericarp in these three lines were distinct while in the CrtZW.DET1 line rarely A type cells observed and the outer pericarp cells contained considerable amount of chloroplasts (see Figure 4-24). This increase in the number of cells contained more chloroplast was the most important reason for the ketocarotenoids elevation.

In summary at the MG stage alteration in photosynthesis and plastid biogenesis of CrtZW.DET1 and the higher intensity of chloroplasts leads to two times higher numbers of chloroplast in CrtZW, which contribution of more numbers of cells containing higher chloroplasts conducted to 7 and 2.6 times increase in total chlorophylls and ketocarotenoids respectively to CrtZW.

In chromoplast biogenesis no appearance of new plastid reported that confirms chromoplasts derived from pre-existing chloroplasts (Pyke and Howells, 2002; Waters et al., 2004).The alterations and increase in carotenoids in DET1 varieties were mainly the effect of *DET1* gene on chloroplast-containing tissues and its consequences on chromoplasts at ripe stage (Enfissi et al., 2010; Davuluri et al., 2004). The higher number of chromoplast and their intensity in CrtZW.DET1 at ripe stage to CrtZW, correlate with CrtZW.DET1 high level of chloroplast and also their intensity at MG stage.

#### 4.7.1.2 More globular chromoplasts and increased bioavailability of ketocarotenoids in CrtZW.DET1

Different forms of chromoplast observed under microscope; in lines containing non endogenous carotenoids mainly spherical types of chromoplast observed while in other lines both forms of spherical and crystalized forms observed frequently. In tomato (Rosso, 1967, 1968), papaya (Schweiggert et al., 2011), carrot (Maass et al., 2009) and mango (Vasquez-Caicedo et al., 2006) the storage of endogenous carotenoids in crystal-like chromoplasts have been reported. Transgenic carrots (Jayaraj et al., 2008), arabidopsis (Mann et al., 2000) and tobacco (Stålberg et al., 2003) producing ketocarotenoids were mostly devoid of crystal forms that implies the storage of their esterified forms within lipid droplets or plastoglobules. The accumulation of ketocarotenoids in alga depends on an active biosynthesis of fatty acids within cells (Schoefs et al., 2001). These data (see section 4.5) correlate with

our spherical forms of chromoplasts in ketocarotenoids containing lines that suggests their lipid dependent storage of nonendogenous ketocarotenoids mainly in lipid droplets and also other endogenous carotenoids in the pre structured spherical chromoplasts. It seems accumulation of ketocarotenoids extend the capacity of cells for storage in globular chromoplasts rather than crystals but more supporting analysis is needed to demonstrate it.

The bioavailability of carotenoids and ketocarotenoids is an important aspect of them, which depends on many factors. In general oxygenated forms of carotenoids are more ready to be absorb into lipid micelle (the ones in human gastrointestinal tract) in comparison to non-oxygenated ones (Yeum and Russell, 2002). On the other aspect, it has been shown that the bio accessibility of globular chromoplasts were higher than crystalized ones (Schweiggert et al., 2012). Considering these points, the line of CrtZW.DET1 contained considerable levels of oxygenated ketocarotenoids in one hand and mainly sequestrated them in form of globular chromoplasts which, made this line a high bioavailable line in comparison to other ones.

#### 4.7.1.3 Ultrastructure changes within plastids for sequestration of more metabolites

In addition to cellular and plastidal characterisations, for a deeper insight to sub-plastidal changes within cells, fractionation analysis was performed. The overall changes of thylakoid and envelope membranes at MG stage and also appearance of the third sharp peak at ripe stage in CrtZW.DET1 (see Figure 4-21), which correlate the higher amount of chlorophylls and pigments and also the higher intensity of plastids in CrtZW.DET1 to CrtZW and Control lines, confirm the overall alteration and expansion in membranes of plastids. These extended membranes have the main role in sequestration of increased metabolites (e.g. seven time increased amount of chlorophylls to CrtZW) in CrtZW.DET1 line.

Plastoglobules are derived from thylakoid membranes by a membrane-blistering mechanism (Austin et al., 2006). Within chromoplast biogenesis from chloroplasts an elevation in the number and size of plastoglobules occur (Harris and Spurr, 1969). Plastoglobules are frequently found in many fruits and flowers and acts as storage vesicles for varieties of carotenoids (Howitt and Pogson, 2006). Larger and more frequent plastoglobules observed in transgenic plants contained more carotenoids for instance in carrots (Vasquez-Caicedo et al., 2006; Jayaraj et al., 2008). These storage

structures (plastoglobules) increased in CrtZW.DET1 and CrtZW lines respectively in comparison to the control line (section 4.3). The total metabolite stored in plastoglobules of CrtZW.DET1 was also higher than CrtZW. But considering each line separately, the percentage of metabolite CrtZW line stored in its PGs from the total amount produced was higher than CrtZW.DET1. For instance CrtZW 11% of its esterified ketocarotenoids stored in plastoglobules while CrtZWDET1 only 4% of them sequestrate in plastoglobules at ripe stage (section 4.3), although still the quantity of metabolite in plastoglobules of CrtZWDET1 was higher to CrtZW because of total increased production of metabolites in CrtZWDET1 line. The effect of *DET1* gene resulted on more available capacity of membranes structures in CrtZW.DET1 line for sequestration of isoprenoids while in CrtZW line the nonendogenous products needs to be accumulate in storage structures of plastoglobules. This observation confirms the expansions in membranes of CrtZW.DET1 in result of *DET1* gene to CrtZW.

#### 4.7.1.4 Increased level of α-tocopherol antioxidant in CrtZW.DET1; plastoglobules may also helps for its production

As the down regulated *DET1* gene plants mimic the molecular response of high exposure to light the increased levels of different bioactive compounds with antioxidant activity have been reported as follows: carotenoids, flavonoids, phenylpropanoids, tocopherols, and ascorbic acid (Enfissi et al., 2010). The 2-fold increased level of tocopherol in DET1 line (TFM7 promoter) accompanied with increase in their transcripts (Enfissi et al., 2010).The considerable increased level of α-tocopherol in CrtZW.DET1 was seen at all of the stages of ripening (2.6, 3.4 and 4.1 fold increase to CrtZW line at MG, 3 dpb and ripe stages respectively (section 4.2.4.1). α-tocopherol acts as an antioxidant by protecting membrane lipids from oxidations that can also delaying senescence (Camara et al., 1983) Specifically tocopherols are important protector in front of oxidative damage to the thylakoid membrane (Havaux et al., 2005; Kanwischer et al., 2005). In CrtZW.DET1 line with noticeable increased amounts of membrane these elevated level of α-tocopherol may helps and act for protection of these membranes from oxidation by pro-oxidants. Also it is suggested that high quinone and α-tocopherol in plastoglobules could help to stabilize the epoxide against oxidation (Ytterberg et al., 2006).

Most of proteins of α-tocopherol biosynthesis pathway were detected in tomato chromoplasts (Barsan et al., 2010) and envelope membrane is the localized place for biosynthesis of α-tocopherol (Arango and Heise, 1998). Plastoglobules participation in some metabolic pathways also reported (Bréhélin and Kessler, 2008). For example both chromoplast and chloroplast plastoglobules harbour a tocopherol cyclase responsible for production of γ-tocopherol that are actively involved in α-tocopherol synthesis (Kessler and Vidi, 2007; Ytterberg et al., 2006) and remarkable existence of α-tocopherol in plastoglobules in CrtZW.DET1 (18% and 14% at MG and Ripe stages respectively) could be in result of this fact that plastoglobules can help in the increased production and storage of tocopherols.

#### 4.7.1.5 Why lycopene as the main accumulated carotenoids barely detected in plastoglobules?

Lycopene is the most abundant carotenoids in tomato fruits. In CrtZW.DET1 line lycopene was 74% of total produced carotenoids and ketocarotenoids. But surprisingly negligible amount of it was stored in plastoglobules while plastoglobules are believed to be designated cellular storage structures. This fact can be considered from a different view:

The first point is that some carotenoid biosynthesis enzymes consist of ζ-carotene desaturase (ZDS), lycopene β-cyclase (LYC-B or CYC-B), and β-carotene β-hydroxylases (CrtR-B1 & B2), exists and active in plastoglobules and specifically LYC-B was identified in large amount which, this suggests that PGs in chromoplasts could have a particular function in carotenoid biosynthesis, in addition to the carotenoid storage/sequestering (Ytterberg et al., 2006). The high activity of cyclase enzymes and alteration of lycopene to β-carotene could be an explanation to low amount of lycopene in plastoglobules.

The second reason for this observation could be the fast rate of production of lycopene (Trudel and Ozbun, 1970; Wu and Kubota, 2008). It is shown that the rate of lycopene accumulation was 3 to 4 times higher than chlorophyll degradation (Egea et al., 2011). This fast production of lycopene mainly from some days post breaker to 7-8 days post breaker result in sequestration of lycopene in its biosynthesis place in forms of mainly crystalloids rather than globular. As it is shown that majority of the carotenoids of tomato fruit are sequestrated mainly in form of lycopene crystalloids within membrane-shaped structures (Harris and Spurr, 1969), this fast production

could be another explanation for approximately absence of lycopene in plastoglobules.

Thirdly, lipid alterations did not show significant changes in our lines (Table 4-16) although more specific experiments are needed on different type of lipids to clarify this point in detail. Considering this fact, and as it is reported before, esterified forms of carotenoids and lycopene did not detected in tomato (Ralley et al., 2004) while many of stored carotenoids are in their esterified form in plastoglobules in other plants like mango, marigold, pepper and apple (Howitt and Pogson, 2006; Vasquez-Caicedo et al., 2006) this exception could be the third reason that lycopene are not abundant in plastoglobules; because esterified form of pigments are more likely to sequestrate in plastoglobules which correlate with our fractionation result that considerable percentage of esterified ketocarotenoids sequestrate in plastoglobules rather than the un-esterified ones.

#### 4.7.1.6 Why lutein and β-carotene are hardly detectable in ketocarotenoid produced lines?

In pigment analysis lutein was detected in very low amounts in comparison to control line in *CrtZW* gene containing lines. At ripe stage in CrtZW.DET1 line lutein decreased 3 and 10 times in comparison to control and the parental line of DET1 respectively while no lutein was detected for CrtZW (see section 4.2.4.1). This reduction of lutein at first is likely in result of active production of ketocarotenoids in CrtZW.DET1 and CrtZW lines from MG stage. Lycopene synthesis is used mainly for production of β-carotene for ketocarotenoids accumulation rather than producing α-carotene and lutein. In the second step, considering the high usage of β-carotene, it also can be considered that β-carotene could also synthetized reversely from precursors like lutein and zeaxanthin (Misawa, 2009). These two points can justify the decreased level of lutein in lines containing *CrtZW* genes.

Decreased level of β-carotene observed in CrtZW.DET1 and CrtZW lines at all of the stages. This carotenoid was not detected in both *CrtZW* gene-containing lines at MG stage while at ripe stage just in CrtZW.DET1 line observed. In result of *DET1* gene enhancing effect, the β-carotene level reached to 0.5 times of control line (see section 4.2.4.1), which is a considerable result in CrtZW.DET1. This decrease in β-carotene level also have been reported by other studies introducing β-carotene hydroxylase and β-carotene ketolase in different plants such as arabidopsis, tobacco, carrots and etc.

(Davison et al., 2002; Yu et al., 2007; Götz et al., 2002; Jayaraj et al., 2008). This decrease in β-carotene reported as a limiting factor for ketocarotenoids accumulation (Stålberg et al., 2003; Morris et al., 2006). This limitation is because of β-carotene role as the first precursor of ketocarotenoids production in the pathway. Specific strategies for increasing the level of β-carotene in the plants could be a good solution for this limitation. For example a genetic cross between high β-carotene varieties with ketocarotenoids varieties can help improvement of β-carotene level. In the research group the genetic cross of CrtZW.DET1 with high β-carotene lines is being undertaken for more improvement of ketocarotenoids production (see section 6.1.2.1.1).

#### 4.7.1.7 Expression alterations in carotenoid biosynthesis genes

For a better understanding about the level of transcripts of carotenoid pathway, the expression of some important genes assessed at two stages of ripening (3 dpb and ripe) using RT-qPCR. In DET1 varieties carotenoid increase was reported in result of plastid parameter increases rather than alteration in carotenoid pathway genes expression because a similar pattern in their expression changes was not observed between DET1 lines with different promoters (Enfissi et al., 2010). In CrtZW.DET1 line a decrease in expression of *DXS* and *PSY1* observed at 3 dpb in comparison to control but similar to DET1 varieties (Enfissi et al., 2010), the overall increase of biomass and precursors in this line covers the decrease in transcripts and results in higher production of carotenoids and ketocarotenoids to control line.

In many of fruits and flowers of higher plants such as red pepper and carrot, β-carotene is a final product. In these plants lycopene as a precursor for synthesis of β-carotene and xanthophylls produced in minute quantities (Nguyen and Schwartz, 1998). But in tomato fruit lycopene counts as the final products, which accumulate in large quantities. At a metabolic level an increase in precursors like phytoene and phytofluene and decrease in β-carotene level is detectable (Nguyen and Schwartz, 1998; Barsan et al., 2010). These metabolite changes control by alterations happen in expression of carotenoid genes during ripening; The related mRNAs for downstream reactions of lycopene (β-carotene cyclases and β-carotene hydroxylases) were strongly downregulated during fruit ripening (Ronen et al., 1999; Barsan et al., 2010). This low level of lycopene cyclization and hydroxylation contributes to high sequestration of lycopene (Bian et al., 2011). Another interesting observation was the

alternative tissue specific expression changes of β-carotene cyclize genes at 3dpb. Which means overexpression of leaf specific cyclase *LCY-B* increased in tomato fruits instead of fruit specific transcript of *CYC-B*. It can be explained by the fact that transcription of carotenoid genes in a specific tissue is strongly under regulation on that tissue and the alternative tissue genes, which does not usually express in that tissue, undergoing the desired alterations and are not under the same regulatory mechanism (Fray et al., 1995).

The two late ripening lines of CrtZW.DET1 and CrtZW have active transcripts at ripe stage. An increase of *DXS*, *PSY1* and *PSY2* transcripts (just in CrtZW.DET1 at ripe) leads to high production of phytoene and phytofluene (up to 2.6 and 4.3 times increase to control) as precursors of lycopene. Also strong downregulation of *CYC-B* in these two lines, severely decline the biosynthesis of downstream metabolites including ketocarotenoids (see Figure 4-26 & Figure 4-22). For instance, the increase of ketocarotenoids accumulation from 3dpb stage to ripe stage was 0.1 times of MG to 3dpb period while this increase for normal carotenoids (mainly lycopene) had 20 times elevation. If only production of ketocarotenoids and not normal carotenoids are targeted, harvest of fruits at 3 dpb suggested as the ketocarotenoids level is enhanced to high levels and a dramatic increase in the production of lycopene has not been initiated yet. But prominent production of lycopene is the end added value for CrtZW.DET1 line, considering the excellent antioxidant property of lycopene (Di Mascio et al., 1989). Production of lycopene in CrtZW.DET1 is 2.5, 1.3 and 1.5 times higher to control, CrtZW and DET1 lines respectively. Other than increased *PSY1* in both of DET1 and CrtZW.DET1 lines, a 2 times increase in *PSY2* expression just in CrtZW.DET1 in addition to highly downregulated CYC-B in CrtZW.DET1 line which was not observed in DET1 line at ripe stage, could explain the highest rate of lycopene production in CrtZW.DET1 line and even to DET1 line.

If this fact is to be considered in parallel with 2.6 times increase of ketocarotenoids in CrtZW.DET1 line, this is a noticeable improvement for both of endogenous carotenoids and nonendogenous ketocarotenoids.

In the two lines of DET1 and control line which follow the normal process of ripening (ripe in 7 days post breaker), higher expression of *DXS* (only in control) and *PSY1* at 3 dpb alongside with low levels of *Lycopene cyclases* transcripts (see Figure 4-26 & Figure 4-22) follow the normal reported process of ripening in these two lines (as described above) which confirm the start of accumulation lycopene as final product

from 3dpb. But in two lines of CrtZW and CrtZW.DET1 with 18 days delay in ripening the pattern is different. With active production of ketocarotenoids from MG stage and consumption of produced β-carotene as their precursor, this high demand, result in higher expression of *LYC-B* in these lines (CrtZW and CrtZW.DET1) at 3dpb (+3phen) (see Figure 4-26 & Figure 4-22). This higher expression of *LYC-B* at +3phen which is around 16 days later than normal overexpression of *LYC-B* at 3dpb in wild type varieties also confirms delay in the ripening of these lines (CrtZW and CrtZW.DET1) (see Figure 4-26 & Figure 4-22). Another interesting observation in CrtZW and CrtZW.DET1 lines were the alternative tissue specific expression changes of β-carotene cyclase genes at 3dpb. Which means overexpression of leaf specific cyclase *LCY-B* increased in tomato fruits instead of fruit specific transcript of *CYC-B* (see Figure 4-22). It can be explained by the fact that transcription of carotenoid genes in a specific tissue is strongly under regulation on that tissue and the alternative tissue genes, which does not usually expresses in that tissue, undergo the desired alterations and are not under the same regulatory mechanism (Fray et al., 1995).

Expression of *CrtW* gene in CrtZW.DET1 was lower to CrtZW although the production of ketocarotenoids was 2.6 times higher in CrtZW.DET1 to CrtZW. This also correlate with the fact of plastid-base increases in metabolites rather than alteration in carotenoid pathway genes expression in DET1 varieties (Enfissi et al., 2010). Expression of *CrtW* gene did not show any difference in CrtZW.DET1 line to CrtZW at ripe stage that correlate with the observation that nearly the production of ketocarotenoids stops at ripe stage (see Figure 4-26 & Figure 4-22).

As expected the level of expression of *DET1* gene in 3 dpb was lower in two lines of DET1 and CrtZW.DET1 which confirm previous studies and its downregulated form at MG to 3dpb (Davuluri et al., 2004, 2005; Enfissi et al., 2010). As downregulation of *DET1* gene did not happened in all stages of ripening in DET1 varieties (Enfissi et al., 2010), expression of *DET1* gene did not show any changes to control at ripe stage in CrtZW.DET1 and DET1 lines in our analysis as well (see Figure 4-26 & Figure 4-22).

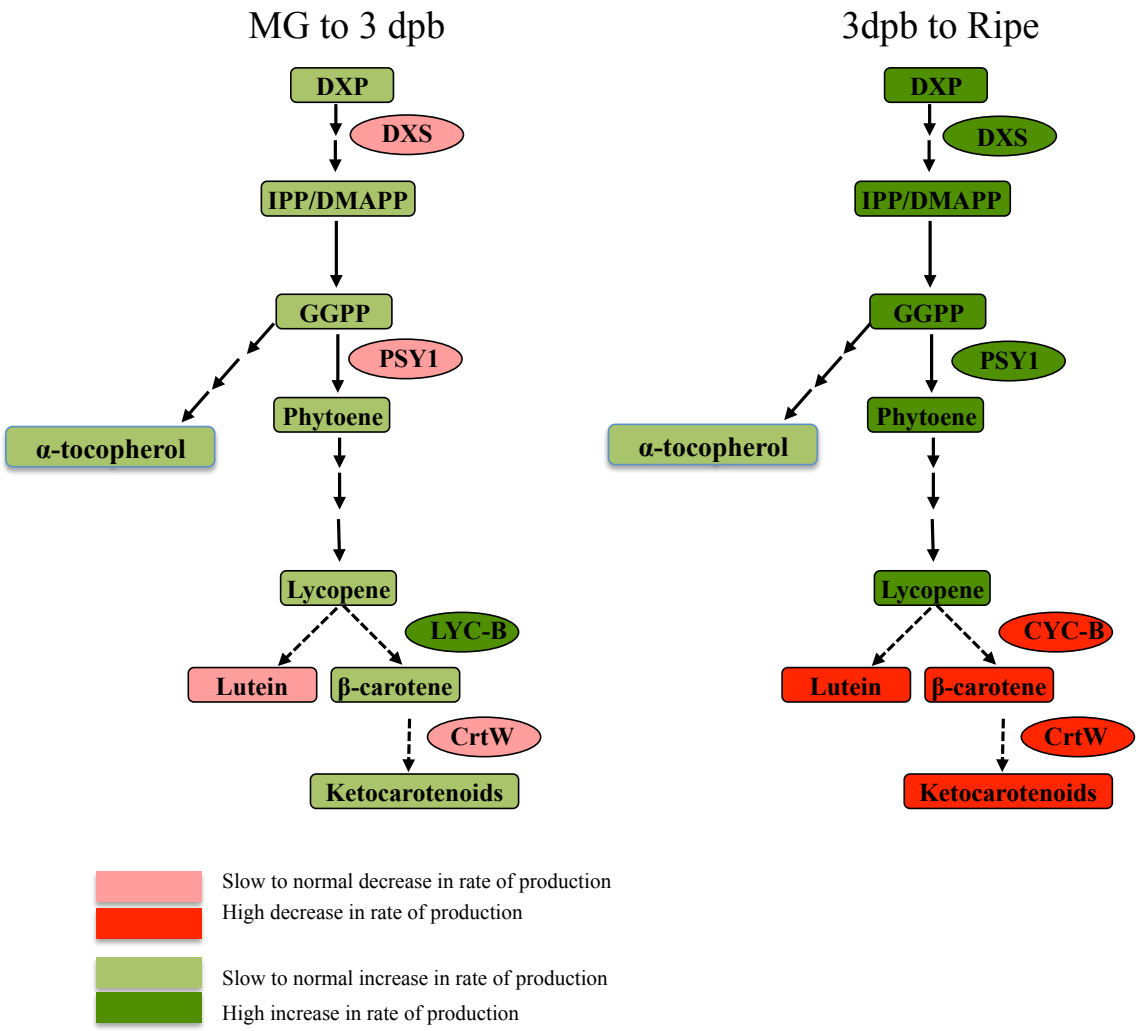



Figure 4-26 The schematic strategy of metabolite production and transcript regulation for production of ketocarotenoids and lycopene from MG to ripe stage in *CrtZW* gene containing lines.

Most of ketocarotenoids yields produced by the 3 dpb via active ketocarotenoids pathway; from 3 dpb to ripe stage cells focus on production final indigenous product (Lycopene) by downregulation of its downstream enzymes within pathway. Data are shown based on carotenoid transcript and metabolite levels representing the rate of metabolite production or the activity of enzymes. Dashed arrows indicate existence of some intermediate steps between the two metabolites, which are not shown, to simplify the pathway DXP: 1-deoxy-D-xylulose 5-phosphate, IPP, isopentenyl pyrophosphate; DMAPP, demethylallyl diphosphate; GGPP, gerenaylgeranyl diphosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; PSY-1, phytoene synthase-1; CYC-B, β-lycopene cyclase; CrtW, β-carotene ketolase; For more detailed expression levels also see Figure 4-22.

#### 4.7.1.8 Other metabolite improvements resulting from the downregulated *DET1* gene in CrtZW.DET1 line

In addition to cellular changes, combination of downregulated *DET1* gene and *CrtZW* has some influential effects on the metabolome as well, demonstrated by metabolite

profiling. As the effect of downregulation *DET1* gene, an overall improvement in biomass of metabolites observed. Theses improvement are expected in an overall pattern within cell metabolites as also previously reported (Enfissi et al., 2010). For example glucose-6-phosphate which is involve in glycolysis, effect the TCA cycle and consequently result an overall increase in primary and secondary metabolites such as amino acids and carotenoids respectively (see Figure 4-17). Glucose-6-phosphate indicated an indefinite increase in 3 dpb and ripe stage in CrtZW.DET1 to CrtZW (see Table 4-16). This overall improvement also signified in remarkable increase of variety of amino acids. For instance serine, proline, glutamine and aspartic acid represent growth at all of stages of ripening (see Figure 4-17). The second group of metabolite altered in a favourable manner was carotenoids as explained in section 4.2.4.1. It is worth mentioning that other than carotenoids with their prominent antioxidant effects, some other antioxidant metabolites also increased; for instance ascorbic acids from organic acids and proline from amino acids (Table 4-16). The PCA data (see Figure 4-16) demonstrate that the significant differences among metabolites are robust enough for separate clusters of the 4 lines (Control, DET1, CrtZW and CrtZW.DET1). The main group of compound, which made shift in loadings, was amino acids as the main altered group of primary metabolites.

### 4.7.2 *APRR2-Like* and *CrtZW* genes does not result in ketocarotenoids and other metabolites improvement

The overexpression *APRR2-Like* gene in the Microtom variety represented more plastids numbers, area and chlorophyll content at MG stage. The increase in carotenoids level at 7 dpb were approximately 1.5 fold in all of the lines which was moderate (Pan et al., 2013). The new crossed genotype of CrtZW.APRR2 created, aimed for improvement of ketocarotenoids production. On the contrary to APRR2-Like line at MG stage, which was expected for the highest level of changes, in CrtZW.APRR2 line a considerable difference was not detected. Surprisingly the chlorophylls content did not present any difference to CrtZW (Mic.MM) or Control (Mic.MM) lines and were considerably lower than the APRR2 parental line. The cell area and chloroplast area also decreased in CrtZW.APRR2 to control line. At MG stage no β-carotene detect in this line and the total ketocarotenoids also did not improved compared to CrtZW (Mic.MM) line. All of the expected improvements about chlorophylls and ketocarotenoids growth did not appear in this genotype at MG

stage. These observations suggest the detrimental effects of *CrtZW* gene on the overexpressed *APRR2-Like* gene. The molecular mechanism underlying this observation could be in result of delayed ABA production.

ABA is synthesised from the carotenoid pathway using β-carotene as a precursor and also downstream xanthophylls like zeaxanthin and violaxanthin in higher plants (Tan et al., 1997; Burbidge et al., 1999). The considerable reduction in the level of β-carotene in CrtZW (Mic.MM) and CrtZW.APRR2 exposed the lack of enough precursors for ABA production on time and result delay in ripening. It is shown that expression of LeNCED1 (a key enzyme in ABA production) is earlier than *LeACS2, LeACS4*, and *LeACO1* (important enzyme in production of ethylene), then it seemed that ABA could act as an upstream regulator before ethylene and be an initial inducer of ripening (Rottmann et al., 1991; Lincoln et al., 1993). Also exposure of tomato flesh and seeds to exogenous ABA treatment, results ABA increase and induces the expression rate of both *ACS* and *ACO* genes which leads to promotion of ethylene synthesis and faster fruit ripening; on the other hand Fluridone or Nordihydroguaiaretic (ABA inhibitors) treatments, delayed fruit ripening and softening (Zhang et al., 2009). The delayed production of ABA and consequently ethylene hormones has been shown in CrtZW.DET1 line (see sections 5.2.1.1, 5.2.1.2 and 5.3.2), which was similar to the delayed ripening phenotype of CrtZW.APRR2 line . This can propose the mechanism leads to late ripening in in CrtZW.APRR2 line is different to CrtZW.DET1 line.

This delay in ABA production could also explain decrease of cell size and absence of APRR2 action on increasing the plastids and chlorophyll contents.

In addition to late ripening, delay in ABA production could be responsible for cell deficiencies as it is demonstrated that ABA has some roles not only in ripening but also in plant growth and development (Zhang et al., 2009; Galpaz et al., 2008). *hp3* represent cell deficiency and decrease of fruit weight by 50% of total weight (Zhang et al., 2009; Galpaz et al., 2008). This could justify the decrease in cell size of CrtZW.APRR2 line in result of delayed in ABA production.

*APRR2-Like* gene affects pigmentation and ripening. Overexpressed *APRR2-Like* gene in Microtom represent larger and more numerous plastids with higher chlorophyll content (Pan et al., 2013). *APRR2-Like* overexpressed plants also overexpressed *RIN*, *ACO* (ACC oxidase), and *PG* (polygalcuronase) which are important ripening factors (Pan et al., 2013). It is also shown that Ethylene can

regulate expression of *RIN* and its target transcription factors effecting ripening (Fujisawa et al., 2013). This fact suggests that the effect of delayed ethylene production (in CrtZW.APRR2 line) on *RIN* or directly on *APRR2-Like* and do not let them to be overexpressed which stopped the effect of *APRR2-Like* gene on more plastid biogenesis and increasing pigment content. Decrease in overexpression of *APRR2-Like* gene in CrtZW.APRR2 compared to APRR2 line at 3dpb and no difference at *APRR2-Like* gene level at ripe stage in CrtZW.APRR2 compared to APRR2 line (see Figure 4-23) correlate to this hypothesis (affect of delayed ethylene production on *RIN* or directly on *APRR2-Like* stopping the effect of *APRR2-Like* gene on more plastid biogenesis and increasing pigment content) which confer this decrease in *APRR2-like* transcripts continued till +3phen stage which is equal to 11 days post breaker in CrtZW.APRR2 line (see Table 4-2) that result in decrease in plastid numbers and pigment content. Moreover, no significant increase in plastid numbers and chlorophyll content at MG stage of CrtZW.APRR2 line also is reported which could be in result of late overexpression of *APRR2-like* gene.

Some other important carotenoid genes were also analysed for understanding the regulation at transcript level. Overall not many changes were observed at transcript level. *DXS* transcript in APRR2 line was higher to control line in 3 dpb and ripe stages (see Figure 4-23). But this increase was not detected in CrtZW.APRR2 line (see Figure 4-23). It is shown that although *DXS* and *ACO1* are regulated indirectly by RIN but their expression has a *RIN* dependent pattern (Fujisawa et al., 2013, 2012). The relation of *RIN* and *APRR2-Like* expression reported in overexpressed APRR2 (Pan et al., 2013) and also in CrtZW.APRR2 which visualized in Figure 4-27. Therefore the over expression of *DXS* in APRR2 line could be in result of *RIN* up regulated affects on downstream genes like *DXS*. The two side effect of RIN and ethylene production on each other also reported (Fujisawa et al., 2013; Kumar et al., 2014) (Figure 4-27). The delay in ABA production in *CrtZW* containing lines could imply its overall inhibitory effect via ethylene, *RIN* and *APRR2-Like* in CrtZW.APRR2 line. Consequently no increase in the expression of DXS was detected in CrtZW.APRR2 line.

The expression of *CYC-B* in two delayed ripening lines of CrtZW.APRR2 and CrtZW (Mic.MM) were downregulated at ripe stage. This is correlate with the fact of downregulation of *CYC-B* for maximize production of lycopene as the final product in tomato fruits (as explained in section 4.7.1.7) (Ronen et al., 1999; Barsan et al.,

2010). In CrtZW.APRR2 other than downregulation of *CYC-B*, *CrtW* expression also highly downregulated for decreasing the consumption β-carotene in ketocarotenoids pathway and accumulation of lycopene as the final product. Similar to this strategy also observed in CrtZW.DET1 line, which described in section 4.7.1.7. The quantitative amount of ketocarotenoids do not show any change from 3dpb to Ripe stage in both lines of CrtZW.APRR2 and CrtZW (Mic.MM) which correlate with the alterations in expression. The only difference that CrtZW.APRR2 represent to CrtZW (Mic.MM) at ripe stage was a slight increase of 1.8 times in ketocarotenoids and α-tocopherol content.

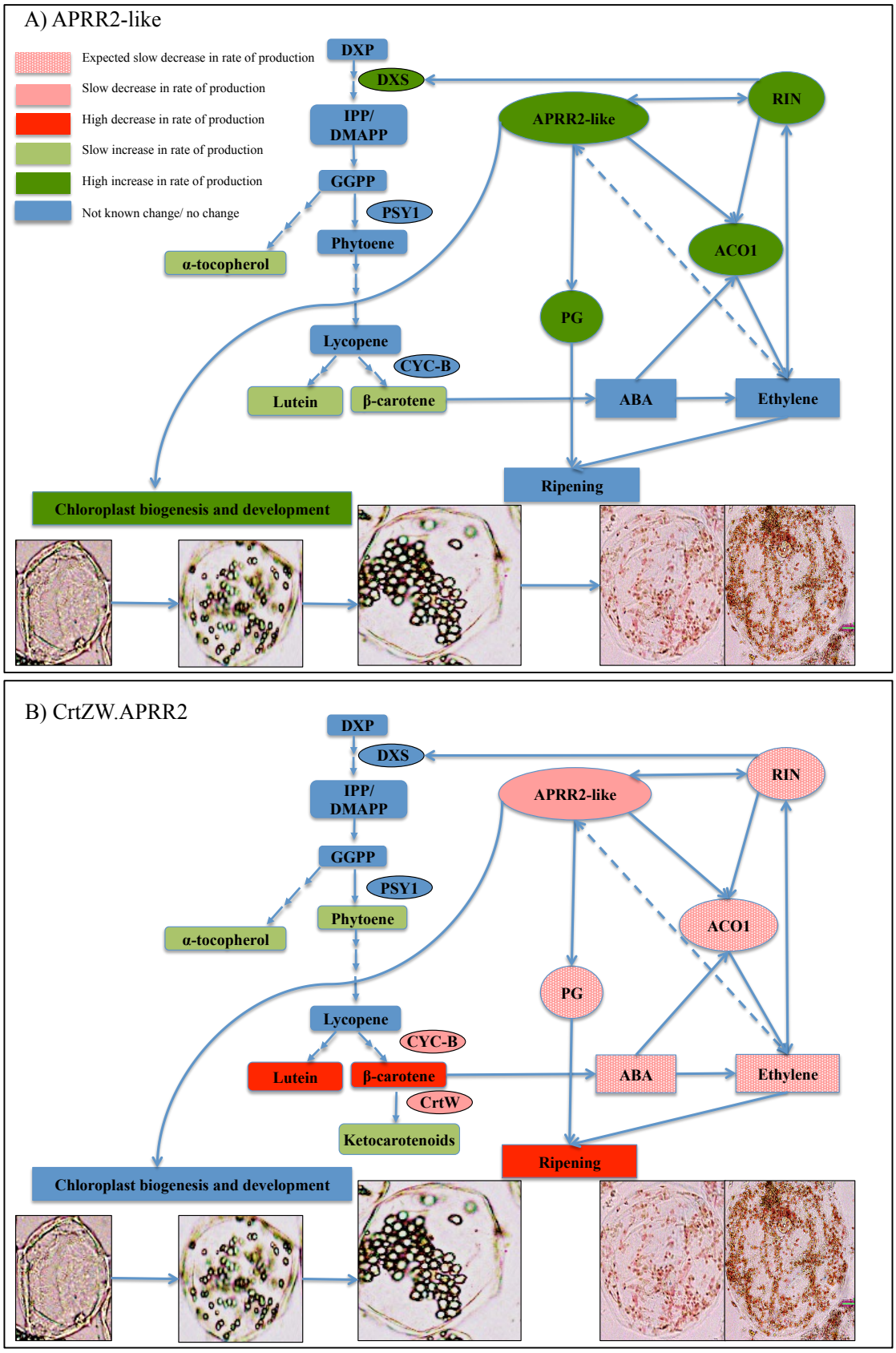



Figure 4-27 The mechanism of *APRR2-Like* gene effect on chloroplast biogenesis and ripening in APRR2 line (A) and the inhibitory effect of CrtZW gene on this process via delay in ABA and ethylene delayed production in CrtZW.APRR2 line (B).

Data are shown based on carotenoid transcript and metabolite levels representing the rate of metabolite production or the activity of enzymes. The transcription factor information relations extracted from (Pan et al., 2013; Zhang et al., 2009; Chernys and Zeevaart, 2000; Fujisawa et al., 2013, 2012). Dashed arrows indicate the proposed relations with no experimental supporting data while solid arrows indicate the relations with experimental supporting data. Two headed arrows represent two side effects and one head arrows represent one side effect. DXP: 1-deoxy-D-xylulose 5-phosphate, IPP, isopentenyl pyrophosphate; DMAPP, demethylallyl diphosphate; GGPP, gerenaylgeranyl diphosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; PSY-1, phytoene synthase-1; CYC-B, β-lycopene cyclase; CrtW, β-carotene ketolase; *ACO1*, ACC oxidase; *PG*, polygalcuronase; ABA, abscisic acid; *RIN*, Ripening inhibitor TF. see result sections 4.2.4.2, 4.4.2, 4.6 and Table 4-13 and Table 4-17 for initial data.

Metabolite profiling was also performed for detection of other metabolite changes. In contrast to the APRR2 line that represents most of changes at MG stage (Perez et al., unpublished), CrtZW.APRR2 line did not show many changes to Control line. In 3dpb and ripe stages an overall decrease in various amino acids and an overall increase in ketocarotenoids observed (see Table 4-17 and Figure 4-19). This observation suggests the degradation of some amino acids for production of enough precursors for consumption in ketocarotenoids pathway, which correlates with absence of chlorophyll and chloroplast changes at MG stage that means no more energy and sugars for consumption in biochemical and biogenesis pathways.

In conclusion CrtZW.APRR2 could not apply the advantages of *APRR2-Like* gene probably in result of decreased level of ABA and ethylene because of active ketocarotenoids production pathway from MG stage. The ABA and ethylene hormone measurement are suggested for this line for confirming this hypothesis. Subsequently a significant improvement in ketocarotenoids level did not observed. This can be concluded that APRR2 gene and CrtZW gene could not act as a good couple of genetic crosses for enhancing the ketocarotenoids production.

### 4.7.3 Overall conclusion

Considering the ketocarotenoids as an important high value compounds with antioxidant effect in organisms, its production by transgenesis has been considered for many years. β-carotene availability could be a limiting factor in the production of ketocarotenoids in many plants like tomato and tobacco (Jayaraj et al., 2008). The strategy used in this chapter for this means was taking the advantages of other transgenic plants with overall increase in chlorophyll and consequently carotenoids levels for providing more precursors for carotenoid pathway and ketocarotenoids production. The two genetic cross of a transcription factor (*APRR2-Like*) and a signal

transduction regulators (*DET1*) were performed with ketocarotenoids producer lines of CrtZW. As it is described in details (sections 4.7.1 and 4.7.2), a positive result was gained from the new genotype of CrtZW.DET1 but not an impressive result from CrtZW.APRR2 line.

This difference was due to the mechanism each gene used for enhancing the metabolites and ketocarotenoids. Downregulated *DET1*, with a negative light signal transduction regulatory mechanism, did not show to have any change on the transcript level of global regulators related to ripening for instance *RIN, NOR* and *CNR*, suggesting *DET1* downregulation was not initiated these cardinal transcription factors (Enfissi et al., 2010). But in contrast, *APRR2-Like* expression was downregulated in all the non-ripening mutants, except CNR suggesting its effect via important ripening transcription factors (Pan et al., 2013). The probable mechanisms of interference of CrtZW gene with these types of transcription factor in result of delayed ABA and ethylene production are explained in sections 4.7.2 and 5.3.2.

Therefore it can be suggested that genes with independent effects from important *RIN, NOR* and *CNR* TFs and other important TFs effecting in ethylene biosynthesis and regulation (Figure 4-28) are effective candidates for combination with *CrtZW* and therefore improvement of ketocarotenoids level.

For instance *GLK* gene could be a good candidate for this means. *GLK* is the closest relative of *APRR2-Like* in tomato but distinct because it lacks the AREAEAA motif conserved in the GLK genes (Fitter et al., 2002). Also cv Microtom harbour the uniform mutation which results in a stop codon in the *GLK2* gene; consequently, *APRR2-Like* cannot be effective via altering the levels of GLK2 protein (Carvalho et al., 2011). *GLK* mediated its effect by activating the expression of PSI and PSII components transcripts from photosynthetic machinery (Powell et al., 2012). Interestingly neither of *GLK1* nor *GLK2* affect general ripening control transcription factors (*RIN, NOR, CNR*, and *TAGL1*) and also no effect was detected on ethylene biosynthesis and signalling genes (Nguyen et al., 2014).

*UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDB1*) also could be a good candidate for improvement of ketocarotenoids synthesis via genetic cross with CrtZW line. *DDB1*, which have counterpart interacts with *DET1* in Arabidopsis, effecting via light signalling pathway (Liu et al., 2004). Its similar effect to *DET1* makes it another potential candidate for this improvement.

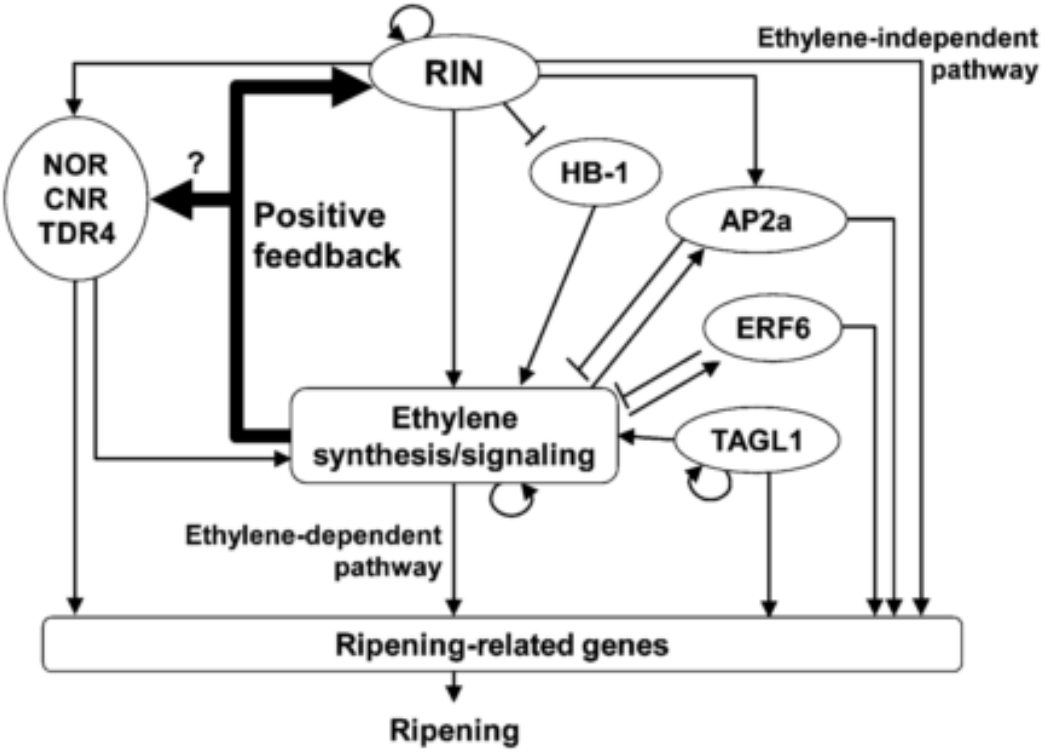



Figure 4-28 "A schematic representation of the proposed model for a regulatory mechanism of tomato fruit ripening, including a mechanism that maintains ethylene levels via RIN and other factors"

"Bold line arrows indicate an ethylene-mediated positive feedback loop that enhances *RIN* expression. It is unclear whether the loop regulates the expression of the other ripening regulators (such as *NOR* and *TDR4*) affected by ethylene during ripening directly or indirectly (via *RIN*). Arrows indicate the direction of the transcriptional regulatory pathways. Blunt- ended lines indicate repression. Circle arrows on *RIN* and *TAGL1* indicate autoregulation and on ethylene indicate autocatalytic ethylene production" (Fujisawa et al., 2013).

Various genes with different effects like genes encoding chloroplast biogenesis or the genes regulating components of light signalling and etc. are potential genetic tools for improving fruit composition and nutritional value if they are used in the right manner. Also with a deep understanding about mechanism underlying and considering their probable conjugation and its bimolecular effects at cellular level and on the final desired product (see section 6.1.2.3).

# CHAPTER 5: UNDERSTANDING MECHANISMS DELAY RIPENING AND IMPROVE SHELF LIFE SPECIFICATION OF CRTZW AND CRTZW.DET1 GENOTYPES

## 5.1 Introduction

The comprehensive characterisations of the CrtZW.DET1 lines, at the metabolite level have been detailed in chapter 4. One of the most important feature of *CrtZW* gene containing lines was their altered shelf life property result in delayed ripening. This advantageous property could lead to a new strategy for improving/extending shelf life both in tomato and other crops. For a better understanding of the underlying mechanisms associated with this delay, further experiments were performed to assess hormonal changes and cell wall remodelling enzymes changes. Cuticle (polymers of lipid and hydrocarbon which impregnated with wax and is synthesized by epidermal cells (Kolattukudy, 1996)) properties such as thickness could also be an important factor on the ripening and shelf life property. The firmness alterations during time and also the weight loss of fruit over time have been recorded for a better appreciation of the delayed ripening arising in the *CrtZW* lines. Collectively, these investigations helped provide a hypothesis to potentially explain the shelf life property and the mechanism underlying CrtZW gene lines with delayed ripening.

As described in section 4.2.1.3 the stages of ripening in CrtZW gene containing lines were different with wild type controls; the Ripe stage (+7 dpb) in wild type is nearly equal to +25 (+7 Phen) as described in section 4.2.1.3 (see Table 4-1); therefor the experiments were continued in late ripening lines till equal phenotypic stage (the phenotypic differences were illustrated in Figure 4-3).

## 5.2 Results

### 5.2.1 Plant hormone changes during developmental stages of ripening

#### 5.2.1.1 Abscisic acid (ABA) hormone alterations

ABA was extracted from three biological replicated samples from freeze-dried samples as described in sections 2.5.1.1 and 2.5.1.2. Three technical replicates of each sample (9 sample per stage; for details of samples see sections 2.1.1 and 4.2.1) ran on LC-MS for ABA measurement and data have analysed with Compass Data Analysis version 4.0. The measurement of ABA and other (hormones and enzymes essays) experiments were continued to +25 dpb for late ripening lines.

The ABA level in Control (T56.MM) line followed the normal wild type ABA expression (Fraser et al., 1995) with a peak at MG stage and the subsequent decreases in the following stages of ripening of 3dpb and 7dpb. But as illustrated in Figure 5-1

in two lines of CrtZW and CrtZW.DET1 producing ketocarotenoids there were a delay in peaks of ABA in comparison to control (T56.MM). In these lines, ABA peaked at 17dpb (phenotypically is equal to 2-3dpb in wild type lines). For discussion see section 5.3.2.1.

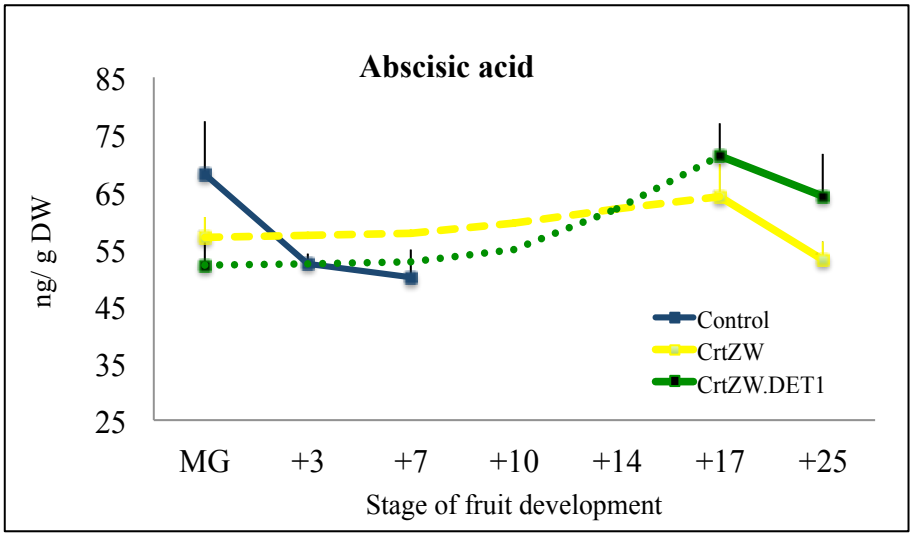



Figure 5-1 Abscisic acid production pattern in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 through stages of ripening

ABA was extracted from frieze-dried samples at 3 stages of ripening with three biological and three technical replicate (minimum of 9 replicate per sample) as described in section 2.5.1.1. The stages of ripening illustrated as MG for mature green and days of post breaker by + sign. For instance, +25 represent 25 days post breaker. Error bars indicate ± SD.

### 5.2.1.2 Ethylene hormone changed pattern

Ethylene measurements were also performed following ABA as an important hormone affecting ripening. Three to four mature green fruits were harvested for each line, weighted and placed in gas-tight jars, sealed and stored for 12 h at room temperature before the experiment. A headspace sample (5 ml) gas was injected to GC gas chromatograph as described in section 2.5.2. Between experiments, fruits were kept *in vitro* under greenhouse condition. The observed delay in ABA peak was also detected in ethylene production as expected; it is proposed that ABA production trigger ethylene synthesis and consequently delay the onset of ripening (Zhang et al., 2009). The characteristic pattern of ethylene production with a peak at 3dpb (see Figure 5-2) was observed in the wild type. But as shown in Figure 5-2 in the two lines of CrtZW and CrtZW.DET1 ethylene released with around 18-20 days delay. The measurements continued till the firmness factor reached to 60-70 % Fff, which is a

typical firmness for ripe fruits see section 4.2.1.2, in CrtZW.DET1 considering the firmness did not decreased till 29dpb, the measurements continued and the second slight peak of ethylene at 27 dpb also detected. The result discussed in section 5.3.2.2.

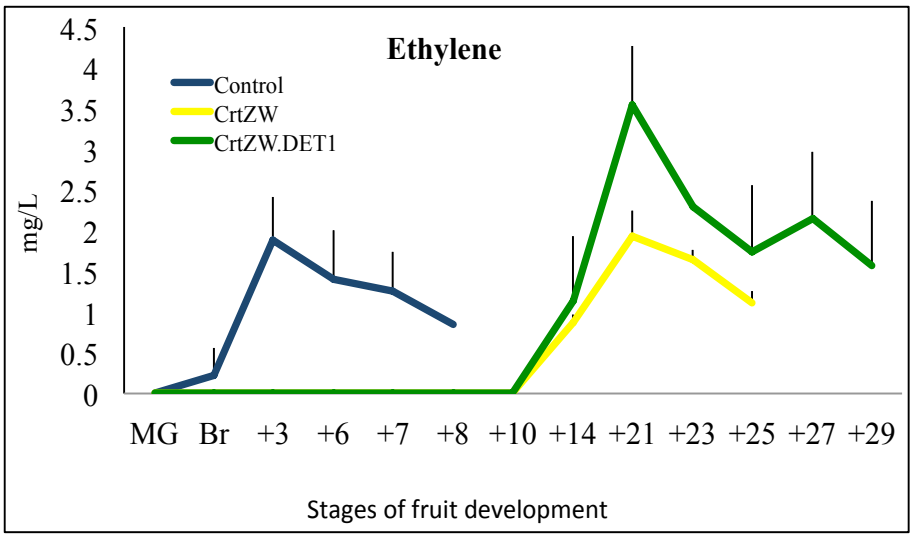



Figure 5-2 Ethylene production pattern in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 through stages of ripening.

Ethylene gas productions of fruits were measured from 3-4 biological replicates as described in section 2.5.2. Mature green fruits was harvested and kept *in vitro* under green house condition. 12 hours before experiment have places in gas-tight jars and sealed with parafilm. The ethylene amounts were detected by GC as detailed in section 2.5.2. The stages of ripening illustrated as MG for mature green and days of post breaker by + sign. For instance +25 represent 25 days post breaker. Error bars indicate ± SD. The number of samples were 3 independent sample with 3 technical replicate (n=9) for each stages of ripening (see section 2.5.2).

### 5.2.2 Screen of important ripening enzyme activities altered in developmental stages of ripening

#### 5.2.2.1 Pectinesterase

Pectinesterase activity was measured from crude enzyme extract (described in section 2.8.1) using a specific colorimetric assay for the enzyme (see section 2.8.4). The test was performed with three independent replicate for each stage of ripening. The activity of this enzyme at 3dpb did not present any changes for CrtZW and CrtZW.DET1 lines in comparison to Control (T56.MM). At ripe stage just CrtZW.DET1 line represent more active pectinesterase to the Control (T56.MM) line (Figure 5-3). For discussion see section 5.3.2.3.

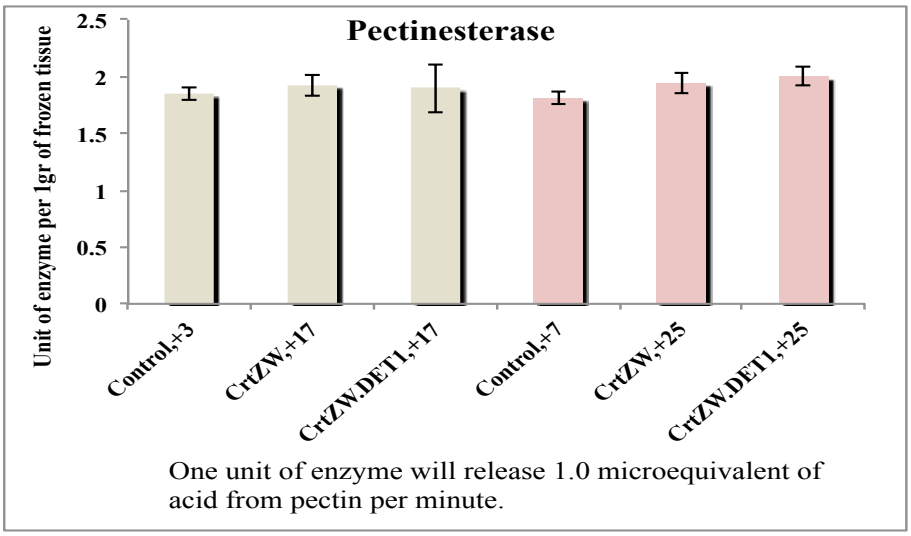



Figure 5-3 The activity of pectinesterase enzyme in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 in two stages of ripening.

Pectinesterase assay have performed as described in section 2.8.4. One unit of enzyme for this essay defined as release of 1 micro equivalent of acid from pectin per minute. Three days post breaker (dpb) in delayed ripening lines is equal to 17 to 19 dpb (cream bars) and 7dpb in delayed ripening lines (pink bars) is equal to 25 dpb as described in section 4.2.1.3. Error bars indicate ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

### 5.2.2.2 Polygalacturonase (PG)

Crude enzyme extract were extracted and used for the specific colorimetric assay of polygalacturonase as described in section 2.8.2. The test was carried out with three independent replicate for each stage of ripening. The activity of this enzyme at 3 dpb and their equivalent stages in delayed ripening lines was considerably low with no difference to control. But at ripe stages the level of enzyme increased; the activity of polygalacturonase is considerably higher in CrtZW.DET1 line in comparison to control and also CrtZW. The enzyme level in CrtZW was significantly higher to control at ripe stage as shown on Figure 5-4. See section 5.3.2.3 for discussion.

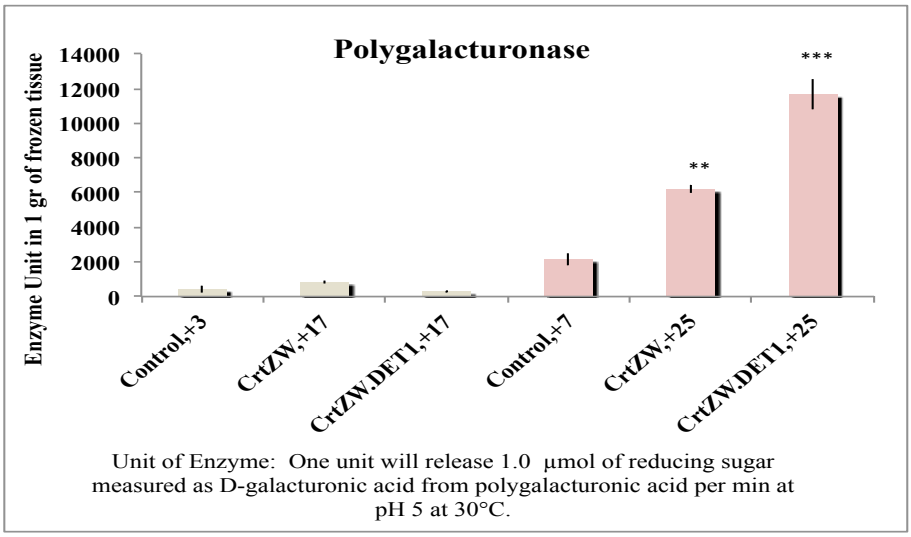



Figure 5-4 The activity of polygalacturonase enzyme in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 in two stages of ripening.

Polygalacturonase assay have performed as described in section 2.8.2. One unit of enzyme for this essay defined as release of 1.0 µmol of reducing sugar measured as D-galacturonic acid from polygalacturonic acid per min at pH 5 at 30°C. Three days post breaker (dpb) in delayed ripening lines is equal to 17 to 19 dpb (cream bars) and 7dpb in delayed ripening lines (pink bars) is equal to 25 dpb as described in section 4.2.1.3. Error bars indicate ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

### 5.2.2.3 β-Galactosidase

This enzyme activity also performed with a specific colorimetric assay as described in section 2.8.3 with three replicate for each stages of ripening. The level of this enzyme in 3dpb were lower in the two delayed ripening lines (CrtZW and CrtZW.DET1) in comparison to control line while at ripe stage only CrtZW.DET1 represent a significant increase to the control line as shown in Figure 5-5. For discussion see section 5.3.2.3.

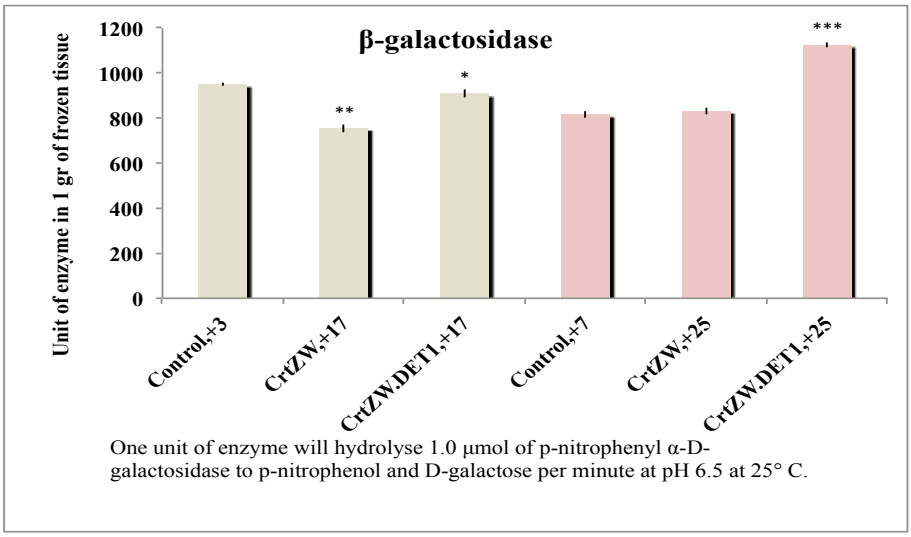



Figure 5-5 The activity of β-galactosidase enzyme in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 in two stages of ripening.

β-galactosidase assay have performed as described in section 2.8.3. One unit of enzyme for this essay defined as hydrolyse of 1 micromole of p-nitrophenyl α-D-galactosidase to p-nitrophenol and D-galactose per minute at pH 6.5 at 25° C. Three days post breaker (dpb) in delayed ripening lines is equal to 17 dpb (+17) (cream bars) and 7dpb in delayed ripening lines (pink bars) is equal to 25 dpb as described in section 4.2.1.3. Error bars indicate ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively.

### 5.2.3 Complementary cell wall component measurements

#### 5.2.3.1 Alcohol-insoluble solids (AISs)

Alcohol-insoluble solids (AISs) were extracted from frozen tomato fruits at three different stages of ripening (see section 2.7.1). This compound has used as the starting material for measurement of other cell wall components.

Typically the amount of AIS is higher at MG stage than other stages of ripening (Table 5-1). 3dpb and ripe stages are the subsequent higher levels of AIS respectively. CrtZW was an exception at ripe stage with higher amount of AIS to 3dpb, which is an unexpected result (Table 5-1). All in all, in CrtZW.DET1 line the amount of extracted AIS was higher in all of the stages of ripening compared to control line.

Table 5-1 The extracted milligrams of Alcohol-insoluble solids (AIS) per gr of frozen tissue in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 in 3 stages of ripening

Alcohol-insoluble solids (AIS) were extracted from frozen samples at 3 stages of ripening with three biological replicate as described in section 2.7.1. MG represents mature green stage. 3 days post breaker in control line and 17dpb in CrtZW and CrtZW.DET1, which phenotypically were equal to 3dpb in wild type line, represented as 3dpb.7 days post breaker in control line and 25dpb in CrtZW and CrtZW.DET1, which phenotypically were equal to 7dpb in wild type lines, indicated as 3dpb. As AIS was used as crude cell wall material for later experiments, one bulk extraction of AISs were performed for each line at each stages of ripening. The result of extracted milligrams of AIS per grams of frozen tissues are reported just for information.

| AISs | MG | +3 | Ripe |
|---|---|---|---|
| Control | 58.95 | 28.45 | 24.07 |
| CrtZW | 58.95 | 28.38 | 37.2 |
| CrtZW.DET1 | 63.46 | 41.67 | 28.51 |

### 5.2.3.2 Uronic acid

Uronic acid was extracted from AIS crude cell wall material. The soluble uronic acid firstly was extracted as described in section 2.7.2 and then underwent for the specific uronic acid colorimetric assay. Similar to AIS result, the overall trend in uronic acid was stepwise, which means three stages on MG, 3dpb and ripe stages represent a subsequent decrease through the stages of ripening respectively.

CrtZW at ripe stage represent an exceptional increase to 3dpb. This difference also has been detected in AISs as well.

Table 5-2 The extracted milligrams of Uronic acids per gr of frozen tissue in three lines of Control (T56.MM), CrtZW and CrtZW.DET1 in 3 stages of ripening

Uronic acids were extracted from frieze-dried samples of AIS at 3 stages of ripening with three replicate as described in section 2.7.2. The data are presented as mean ± SD. MG represents mature green stage. 3 days post breaker in control line and 17dpb in CrtZW and CrtZW.DET1, which phenotypically were equal to 3dpb in wild type lines, represented as 3dpb. 7 days post breaker in control line and 25dpb in CrtZW and CrtZW.DET1, which phenotypically were equal to 7dpb in wild type line, indicated as 3dpb.

| Uronic acid | MG | 3dpb | Ripe |
|---|---|---|---|
| Control | 7.5+/-1.71 | 5.5+/-0.41 | 5.7+/-1.44 |
| CrtZW | 7.2+/-0.18 | 5.0+/-0.43 | 8.9+/-2.23 |
| CrtZWDET1 | 7.0+/-1.62 | 6.1+/-1.25 | 5.4+/-0.22 |

#### 5.2.3.3 Glucuronic acid

The glucuronic acid data were extracted from the metabolomics data for these lines (see Table 4-16). As glucuronic acid takes part in cell wall, its data illustrated again in Table 5-3 for a comprehensive comparison with other changed enzymes and metabolites.

In CrtZW.DET1 the level of this compound was considerably higher to control and CrtZW at 3dpb and ripe stages. This difference between glucuronic acid in CrtZW and CrtZW.DET1 is 1.5 times higher to this difference in Control and CrtZW.DET1 lines.

Table 5-3 The ratios of glucuronic acid changes at different stages of fruit development and ripening in the ZWDET1 line tomato fruits compared to its three derived comparators.

CONT: Control (T56.MM), ZW: CrtZW (T56.MM) and DET: DET1 (T56.MM). Data obtained from GC-MS analytical platform with subsequent statistical analysis. The data are presented as mean ± SD. Student's t-test was performed for finding significant changes, which are shown in bold. P values with the range of $P<0.05$, $P<0.01$ and $P<0.001$ are shown by *, ** and *** respectively. If a metabolite is unique to ZWDET1 at the concentration used is shown as In Inc (indefinite increase); and when is unique to a comparator line at the sample concentration used is shown as In Dec (indefinite decrease) and "-" indicates metabolite was not detectable in both ZWDET1 and the comparator at the sample concentration used.

| | MG | **3dpb** | **7dpb** |
|---|---|---|---|
| ZWDET/CONT | 2.55±0.5 | **2.83±0.1 **** | **1.97±0.3 *** |
| ZWDET/ZW | **In Inc** | **4.15±0.1 ***** | **3.21±0.4 **** |
| ZWDET/DET | - | 3.3±0.1 | 2.78±0.4 |

### 5.2.4 Cuticle thickness was not change in CrtZW.DET1 to wild type control.

Cuticle thickness measurements performed on MG fruits in two lines of Control (T56.MM) and CrtZW.DET1 as described in (section 2.6.2). Three biological replicate with 7-10 measurement through different sections of fruits have performed. The thickness result did not present any statistically difference between Control (T56.MM) and CrtZW.DET1. Which means cuticle thickness is not responsible for the delay in ripening of CrtZW.DET1 line. See section 5.3.2.3 for discussion.

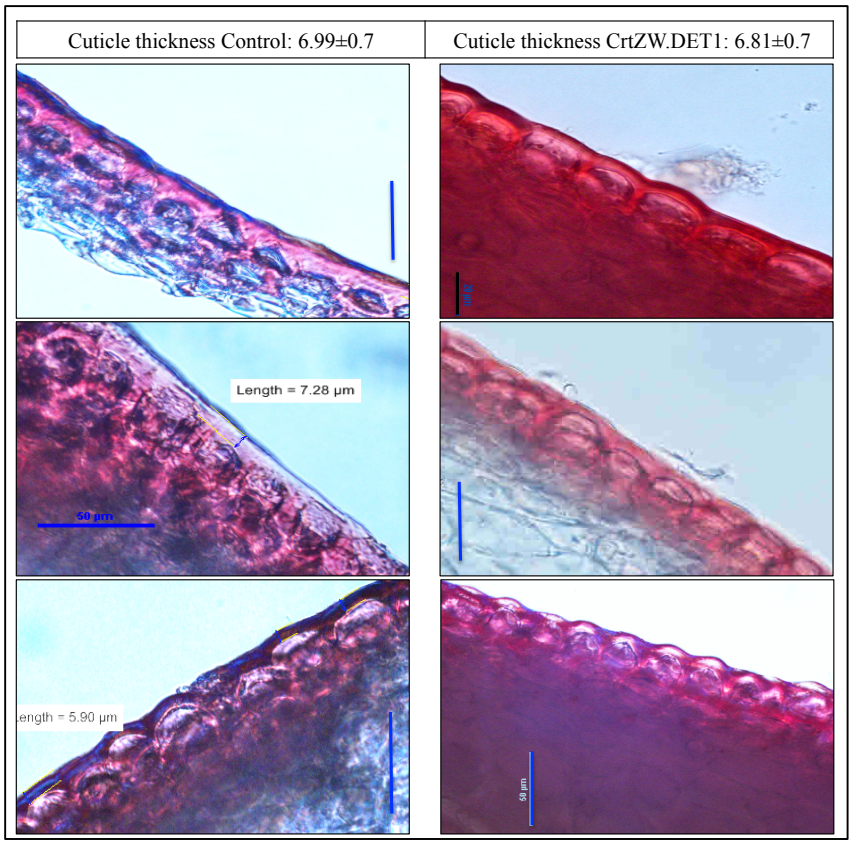



Figure 5-6 Cuticle thickness measurement average and representative pictures of cuticle in two lines of CrtZW.DET1 and its control (MM.T56)

Cuticle thickness measurements performed on MG fruits in two lines of Control (T56.MM) and CrtZW.DET1. Three biological replicate with 7-10 measurement through different sections of fruits have performed. The data are presented as mean ± SD. Student's t-test was performed for finding significant changes; no significant changes detected for the two lines. Three representative images of cuticle measurements illustrated for each line as well. The blue scales bars represent 50 µm and black scale bar indicates 20 µm.

#### 5.2.4.1 Complementary shelf life experiment

#### 5.2.4.2 Firmness changes and weight loss pattern during shelf storage

Firmness measurement were performed under shelf-life experiments; 9 ripe fruits for each line were collected and sterilised as explained in section 2.9. Regular firmness measurement performed and the data plotted as shown in Figure 5-7 for finding the trend of alterations in each line. The slope of alterations in control (MM.T56) line is the steepest one. Considering the duration of firmness loss, Control line looses the firmness in nearly 75-80 days, which is the fastest line. Firmness property in DET1 line was improved by nearly 15 days in the time needed to firmness factor reached to

0% of Fff. The slope of this line is slighter to control and is nearly the same with two late ripening lines of CrtZW and CrtZW.DET1. The time was needed for two lines of CrtZW and CrtZW.DET1 which firmness factor reached to 0% of Fff improved by nearly 50 days more to control line and reached to 140 days post breaker as shown in Figure 5-7. This is a considerable improve in the firmness factor of these two lines. Another notable point was about the difference between CrtZW and CrtZW.DET1 lines. The fruits of CrtZW.DET1 at 25 dpb that harvest from plant are around 20 % Fff firmer than CrtZW, and at day 140 the difference decreased to 10 % Fff difference. This fact confers the slight faster loosing of vigour in CrtZW.DET1 but in overall CrtZW.DET1 line is the best-improved line in terms of firmness.

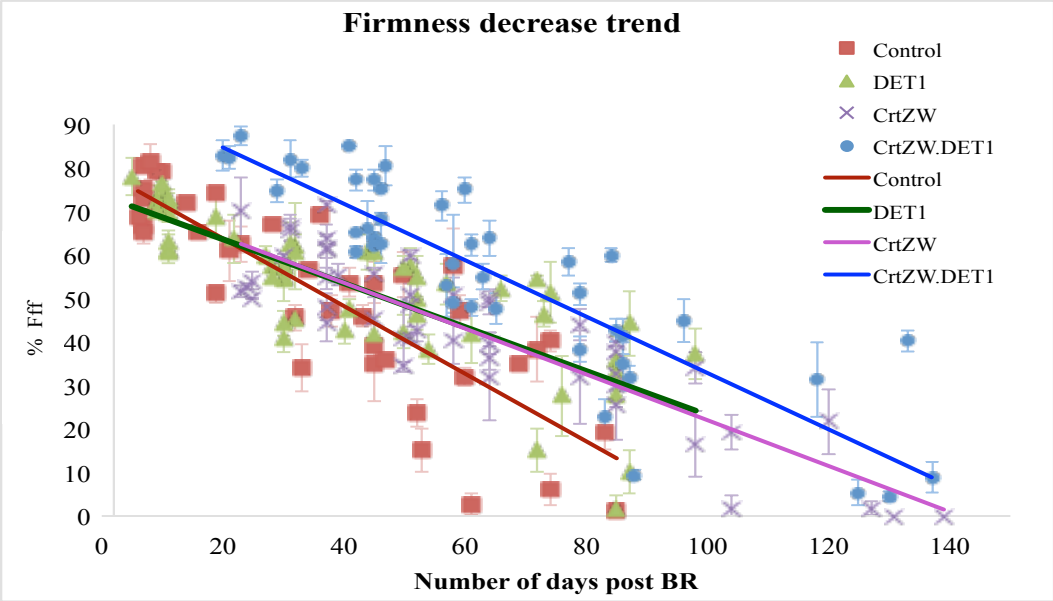



Figure 5-7 percentage of Fruit firmness factor (Fff) alterations by time in CrtZW.DET1 and its derived comparators of DET1, Control (T56.MM) and CrtZW.

The data obtained from minimum of 9 ripe fruits each line. The firmness factor with three replicate performed regularly till the firmness factor reached to zero. The experiment for two lines of Control and DET1 continued approximately 80 days post breaker but for two lines of CrtZW.DET1 and CrtZW continued 140 days post breaker time. The previously sterile fruits stored at 19°C in dark in sterile containers till next measurement (see section 2.9). The result for each measurement represent as a single point of means and Error bars indicate ± SD. The leaner standard curve indicates the trend of alteration in each line. For more detail see section 2.1.2.

Weight measurements also performed under shelf-life experiments with the same 9 ripe fruits used for firmness. Samples weighted regularly and the data plotted as shown in Figure 5-8 for finding the trend of alterations in each line. Similarly to firmness result, the slope for altered weight compared to the control (MM.T56) line is

the steepest one, which is the fastest line losing weight by time. The fruit weight loss in DET1 line was improved, the slope of this line is not has pronounced as the control and is similar to the two late ripening lines of CrtZW and CrtZW.DET1.

The two lines of CrtZW and CrtZW.DET1 with a less pronounced slope to control line lose their weight during time, which is a noticeable improvement in the weight loss of these two lines by time.

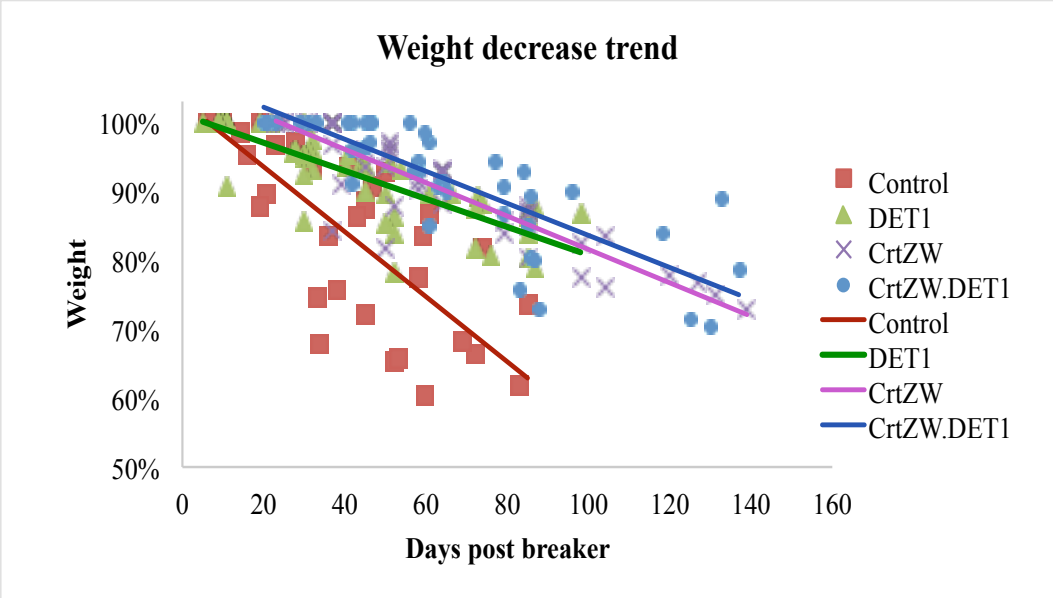



Figure 5-8 Weight alterations by time in CrtZW.DET1 and its derived comparators of DET1, Control (T56.MM) and CrtZW.

The data obtained from minimum of 9 ripe fruits each line. The weight of samples measured regularly till the firmness factor reached to 0% Fff. The experiment for two lines of Control and DET1 continued approximately 80 days post breaker but for two lines of CrtZW.DET1 and CrtZW continued 140 days post breaker time. The previously sterile fruits stored at 19°C in a dark place in sterile containers till next measurement (see section 2.9). The result for each measurement represent as a single point on chart. The leaner standard curve indicates the trend of alteration in each line.

Precise statistical information was extracted from Figure 5-7 and Figure 5-8 to have a insight to the trend of weight loss and firmness changes which represent in Table 5-4. DET1 and CrtZW.DET1 represent the best numbers for weight loss and firmness loss respectively. But collectively considering the high fruit resistance of CrtZW.DET1 (131 days) in addition to its 25% of weight loss in 116 days, made it the most improved shelf life genotype. CrtZW.DET1 line takes the advantage of both of parental shelf life property (CrtZW and DET1) for improving its shelf life.

Table 5-4 Informative report about weight and firmness alterations in CrtZW.DET1, DET1, Control (T56.MM) and CrtZW.

The data extracted and calculated from equation of tread lines in Figure 5-7 and Figure 5-8. Shelf life experiments were performed on three biological and three technical replicates for each line. Weighing and firmness measurements performed regularly and plotted for illustration of Figure 5-7 and Figure 5-8.

| | **Days needed percentage of Fff decreased to 0 %Fff** | **25% weight loss (Days)** | **50% Fff loss (Days)** |
|---|---|---|---|
| Control | 75 | 55 | 51 |
| DET1 | 90 | 126 | 73 |
| CrtZW | 118 | 110 | 71 |
| CrtZW.DET1 | 131 | 116 | 75 |

### 5.2.4.3 Pigment level changes during storage

The samples used for the shelf life experiment were harvested at defined stages of 50, 80 and 130 dpb and freeze-dried for pigment analysis as describes in section 2.9 with 3 replicate for each defined stage. For detecting the pigment alteration trends, the pigments levels different stages were plotted against days post breaker. The important pigments of lycopene, total ketocarotenoids and α-tocopherol were illustrated in Figure 5-9. A clear trend for increase in pigments content was detectable in CrtZW and CrtZW.DET1 in most of shelf life stages, but on the other side the pigment alteration in the two lines of Control and DET1 were nearly constant with slight changes. Ketocarotenoid accumulation continued in CrtZW.DET1with nearly 30 μg/gr DW difference with CrtZW in all of the stages. Production of lycopene also continued at all of shelf life stages of CrtZW line and most of stages of CrtZW.DET1 line (the trend got steady in the last stage), but still quantitatively higher than all the other lines. The most interesting result is about α-tocopherol with its continued increase and its considerable amount in CrtZW.DET1 during the shelf life stages, which indicate the active cellular production of this compound. This aspect could be a value added attribute for this line with increased shelf life extension in addition to more accumulation of high-value compounds during shelf life period as well.

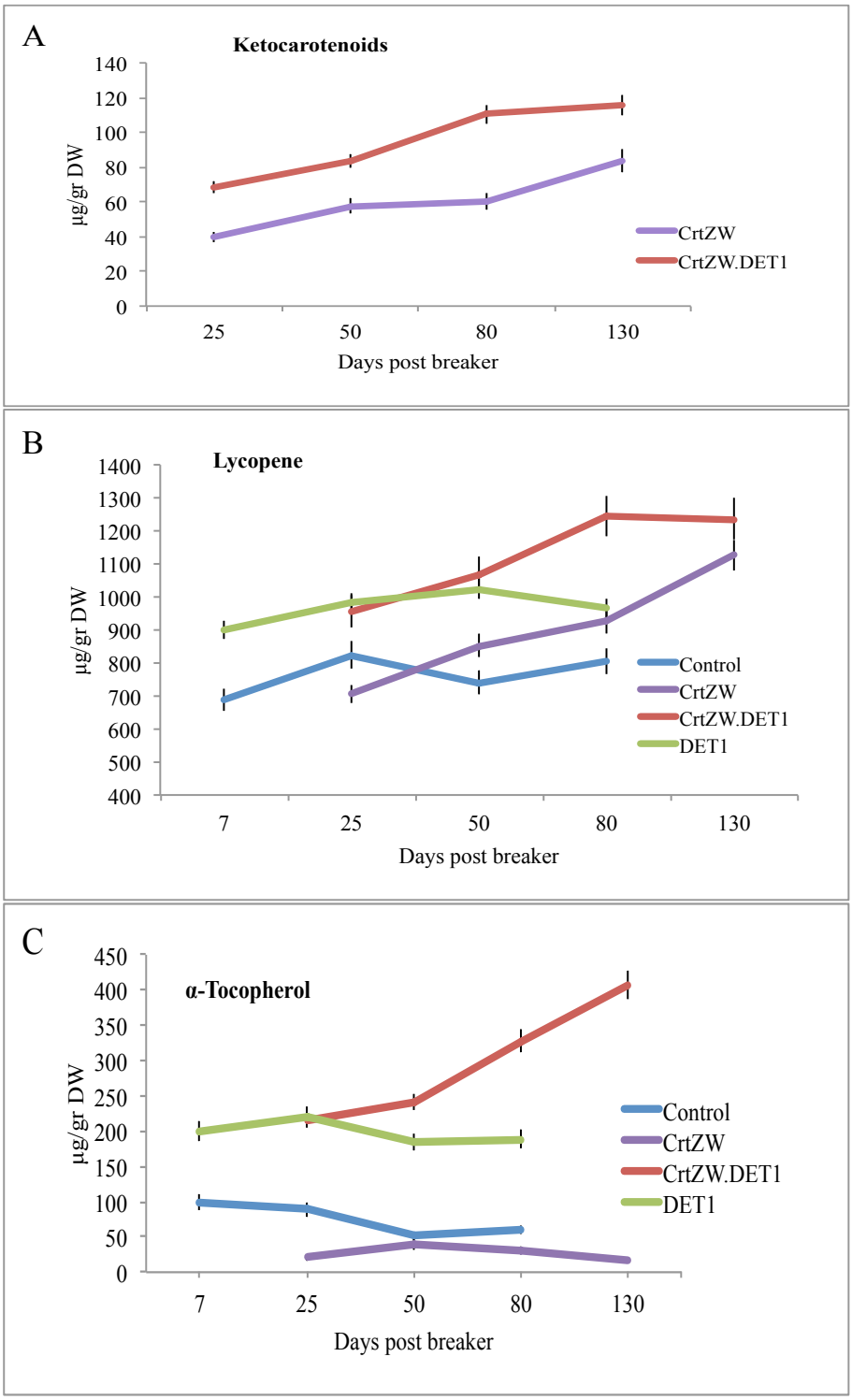



Figure 5-9 A, total ketocarotenoids, B, lycopene and C, α-Tocopherol alterations after ripening by time in CrtZW.DET1 and its derived comparators of DET1, Control (T56.MM) and CrtZW.

The data obtained from minimum of 9 ripe fruits each line. The samples of shelf life experiment harvested in defined stages of 50, 80 and 130 dpb and freeze-dried for pigment analysis as describes in section 2.9. The previously sterile fruits stored at 19°C in a dark place in sterile containers during the experiment (see section 2.9). Means of pigments analysis were plotted for illustrating the trend figures and Error bars indicate ± SD.

## 5.3 Discussion

### 5.3.1 Delay in ripening and improved shelf life capacity of CrtZW and CrtZW.DET1 lines.

Fruit ripening is a complex coordinated event with several regulatory steps in fruits for obtaining their final physiological trait and their specific high value metabolites (Kumar et al., 2014). With the progress of ripening, different alterations occur in different aspects. This study tries to perform relevant experiments for a better understanding about this process in delayed ripening line of CrtZW.DET1. And also its derived CrtZW and DET1 lines with delay ripening and shelf life improvement characteristics. The colour change owing to accumulation of pigments is an important ripening characteristic. The pigment measurements for the ripening steps and also during shelf life storage were performed for these lines (sections 4.2.4.1 and 5.2.4.3). Other changes such as conversion of complicated carbohydrates to sugars resulted in fruit acidity reduction, accumulation of metabolites responsible for flavour/aroma and considerable cell wall changes which leads to softening or dehiscence (Klee and Giovannoni, 2011; Seymour et al., 2013b). Changes in cell wall thickness, plasma membrane permeability, cell wall hydration, increase in intracellular spaces and structural intensity reduction are in result of cell wall alterations (Redgwell et al., 1997); the most apparent compositional changes in cell wall belong to pectin alterations (Smith et al., 1998) which consist of depolymerisation and desertification, solubility increase, lose of sugar containing side chains of cell wall compounds (Huber, 1983b). Cell wall changes mediate by specific cell wall degrading enzymes such as pectinesterases, β-galactosidase and polygalacturonase (PG). In this study the alterations in some of cell wall compounds such as alcohol insoluble solids (AIS), uronic acids and glucuronic acid and also some important cell wall degrading enzymes (pectinesterases, β-galactosidase and polygalacturonase) were detected in Control (T56.MM), CrtZW and CrtZW.DET1 lines. The mentioned processes control by multiple genetic regulators (important TFs such as *RIN, NOR* and *CNR*) and biochemical pathways and hormonal alterations (see section 1.5). As many of these changes observed in the context of hormonal changes, the main hormones control ripening process seems to be ABA and Ethylene (Giovannoni, 2004; McAtee et al., 2013). Some of cell wall compounds and ABA and Ethylene hormone measured in this study.

There are some other ripening-related physiological processes, which play important role in ripening for instance cellular turgor and cuticle thickness. Cuticle is the waxy layer which is consist of cutin polyester and variety of waxes covering the aerial surface of land plants (Jeffree, 2006; Nawrath, 2006). Cuticle is responsible for some of biological functions such as maintaining fruit skin integrity, controlling cuticular respiration, limiting the microbial infections and affecting fruit softening and shelf life (Hovav et al., 2007; Leide et al., 2007; Saladié et al., 2007). The cuticle thickness of CrtZW.DET1 was also compared with the control line in this study as well.

All of the above-mentioned experiments were performed to provide a better understanding about the mechanism underlying this late ripening lines and their shelf life improvement as shelf life affected by many factors such as fruit firmness, cell wall structure and alterations, cuticle properties, cellular turgor (weight loss by time is a good indicator of cellular turgor) (Vicente et al., 2007). It has been shown that approximately 15 and 18 days delay in the ripening of CrtZW, CrtZW.DET1 lines between breaker and ripe stage respectively (see section 4.2.1.3). Moreover considering over ripening, the different trends for firmness and weight loss alterations indicated the firmness and weight decreases were much less in CrtZW, CrtZW.DET1 and DET1 in comparison to the control through the storage period after fruit harvest. In CrtZW, CrtZW.DET1 100% and 110% slower weight loss by time and 39% and 47% Fff improvement in their firmness were detected to control respectively (see Figure 5-7, Figure 5-8 and Table 5-4). In the next sections the probable mechanism underlying delayed ripening and shelf life improvement are discussed.

### 5.3.2 Delay in ABA production could delay ripening via delay in Ethylene peak in CrtZW and CrtZW.DET1 lines

#### 5.3.2.1 Reduced β-carotene, the precursor for ABA

Active production of ketocarotenoids start at MG stage from the precursor β-carotene in *CrtZW* gene containing lines. The lack of β-carotene and its onward pigments in carotenoid biosynthesis pathway confirms the consumption of β-carotene in ketocarotenoids pathway (described in section 4.7.1.6). As a consequence of this property, the lack of ABA hormone precursor (β-carotene) could justify its late production in CrtZW and CrtZW.DET1 lines. As illustrated in Figure 5-1, the control line (MM.T56) represents a peak of ABA at MG stage, which correlate with other

wild type tomato fruits as reported previously (Fraser et al., 1995; Zhang et al., 2009). In late ripening lines of in CrtZW and CrtZW.DET1 ABA peaked 17-18 days post breaker, the stage which process of ripening starts, (see section 4.2.1.3). As illustrated in Figure 5-10 the rate of ketocarotenoids production from this stage onward also declined which can provide enough β-carotene precursor for accumulation of ABA hormone peak at this stage and consequently the subsequent ripening process proceeds. ABA is an important hormone for ripening (see section 1.5.6) and it is shown that any treatment that delay the ABA peak, will delay the induction of ripening and softening in tomato fruits. Expression of the ABA biosynthetic gene (*LeNCED1*) takes place before ethylene genes expression. It is also shown that exogenous ABA treatment increase fruit ripening process, and on the other hand treatment with fluridone or NDGA (ABA inhibitors) delayed fruit ripening (Zhang et al., 2009). Also silencing of a key enzyme in the ABA biosynthesis is *SlNCED1* gene, encoding 9-*cis*-epoxycarotenoid dioxygenase (NCED), represent higher pectin level, enhanced fruit firmness and improved shelf life (Sun et al., 2012b). It is important to note that ABA peak in the onset of ripening were reported in both climacteric (Buesa et al., 1994) and non-climacteric fruits which confer its important role in the ripening process (Leng et al., 2014). It is shown that later start of fruit de-greening in ABA deficient orange mutants (Rodrigo et al., 2003) and also in FaPYL1(a class of cytosolic ABA receptors)-RNA interference (RNAi)-silenced strawberry the inhibition of ABA signalling delayed fruit ripening (Jia et al., 2011).

ABA affects ripening directly and indirectly via triggering the ethylene production. The direct effect of ABA was explained above. The second effect of ABA on ethylene is very important as ethylene plays a key role in later stages of ripening. It is demonstrated that ABA production initiate the climacteric increase in ethylene production and also probably ABA induce ethylene production by regulation of *ACS* and *ACO* gene expression (Zhang et al., 2009). The role of ethylene will be discussed in more details in the next section.

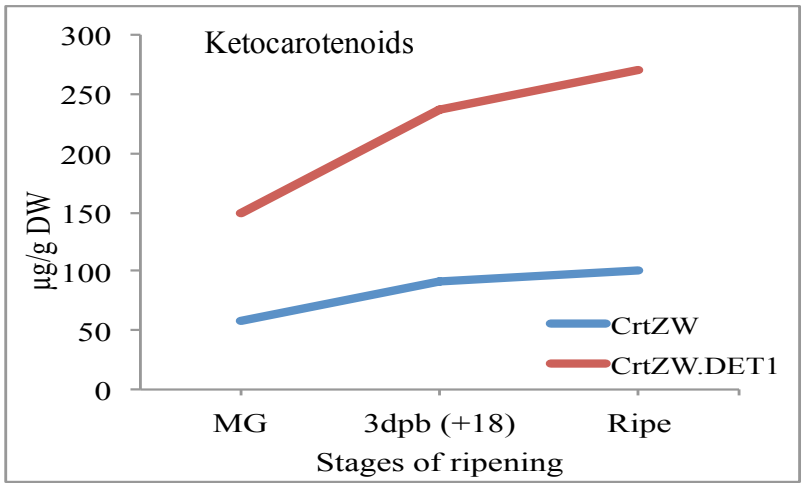



Figure 5-10 Alterations in rate of ketocarotenoids production during stages of ripening in CrtZW and CrtZW.DET1.

The ketocarotenoids data from UPLC (pigment analysis) have plotted during stages of ripening in two lines of CrtZW (blue line) and CrtZW.DET1 (red line). MG, +3 and Ripe represent mature green; 3dpb is equal to 18dpb in *CrtZW* gene containing lines; and Ripe stage which is approximately 28 dpb. The number of samples were minimum of 3 to 4 independent samples with 3 technical replicate (n=9 to 12) for each stages of ripening (see section 2.5.2).

#### 5.3.2.2 Delay in system II climacteric Ethylene peak postponing the ripening in CrtZW, CrtZW.DET1 lines

As described fully in the introduction section 1.5.5 the ethylene production in tomato fruit as a climacteric fruit in the process of fruit ripening related to system II ethylene production. This burst in ethylene is critical for fruit ripening and maintaining the shelf life (Pech et al., 2012). As explained in previous section in *CrtZW* containing lines represent a delay in ethylene production as a result of late ABA accumulation because of lack of its precursor β-carotene. In CrtZW and CrtZW.DET1 lines ethylene level peaked at 21 dpb (equal to 3-4 dpb in normal tomatoes), which is 3 to 4 day after the ABA peak. In normal tomatoes also the difference between ABA and ethylene peaks were the same which correlate with other articles (Leng et al., 2014). This delay in ethylene is the second main reason for delay in ripening which is mediated by ABA delay production. On the contrary to *hp3* ABA deficient mutant (Galpaz et al., 2008), ethylene level in CrtZW did not present a significant difference with control line while in CrtZW.DET1 it is increased approximately two times higher than control. Indeed the first ethylene burst was significantly higher but the firmness did not decrease as expected (see section 4.2.2.3) and the second peak in ethylene occurred at 27 dpb as shown in Figure 5-2. This second peak in ethylene just

occurred in CrtZW.DET1 which could be in result of its stronger cellular cell wall characteristics, which needs more ethylene hormone to complete softening. This hypothesis is discussed fully in section 5.3.3.

Fruit colour changes is closely related to ethylene. The expression of some genes in carotenoid pathway such as *PSY1* is shown to be induced by ethylene. Indicating that production of carotenoids responsible for pigmentation is tightly regulated by ethylene (Kumar et al., 2014). Over expression of *PSY1* in *CrtZW* containing genes lines was at ripe stage, which is after ethylene rise and confirming this fact (see section 4.4.1 and Figure 5-2). Furthermore some ethylene biosynthetic genes such as LeACS2 and some signalling genes for example *SlAP2a* and *SlERF6* were found to affect tomato fruit pigmentation (Karlova et al., 2011; Lee et al., 2012). Fruit pigmentation is not the only field affects by ethylene, but also cell wall degrading enzymes production have a considerable joint with ethylene. Some ethylene-regulated genes such as cell wall degrading genes (polygalacturonase; pectinesterase and pectate lyase) have been studied (Camara, 1994). Some enzymes, metabolite alterations related to cell wall degradation are discussed in the next section.

#### 5.3.2.3 Delayed ethylene production affects cellular enzymes, components and susceptibility

The cell wall composition was described in section 1.5.7.1. Alteration in the cell wall is linked with fruit softening. This softening correlate with the expression of some hydrolase and transglycosylase genes (Huber, 1983a), including transcript and enzyme activity increase of polygalacturonase (PG) (Smith and Gross, 2000), expansion (Exp1) (Brummell et al., 1999a; Cosgrove, 2000) and pectin methylesterase (PME) (Tieman et al., 1992). Ethylene for many years considered as the main ripening inducer as represent different effects on these enzyme activities in the process of ripening. However recently the direct molecular effect of ABA in fruit softening reported (see section 5.3.2.1) (Sun et al., 2012b). In the process of ripening previously suggested that one or two enzymes with hydrolysis effect such as PG or PME control textural alterations in tomato because of their alterations during the ripening (Giovannoni, 2001; Brummell, 2006). But transgenic plants with silenced or downregulated candidate genes (PG or PME) did not support this hypothesis. It seems ripening is more in result of simultaneous effects of multiple such genes; and also many new candidate genes were introduced as well (Brummell, 2006; Powell et al.,

2003; Seymour et al., 2013a). Changes in the activity of these enzymes and also cell wall components are a good indicator of ripening process in fruits. The changes in the activity of some important enzymes measured in CrtZW and CrtZW.DET1 and compared to control.

β-galactosidase, which proceeds galactose release from cell wall polymers, demonstrated a direct regulation by ethylene. Ethylene antagonist treatments of wild type tomato fruits prevent the expression of two endo-β-galactosidases (Lashbrook et al., 1994). The sum of β-galactosidases enzymes level did not represent a considerable difference in the stages of ripening but the individual forms change during ripening (Pressey, 1983). The level of these enzymes in control and CrtZW lines were relatively constant in 3 dpb and ripe stages in correlation with other articles (Rugkong et al., 2010), while in the CrtZW.DET1 represent higher activity at ripe stage (see Figure 5-5). Pectin degrading enzymes such as pectinesterases are the most implicated enzymes in fruit softening. Several pectin remodelling enzymes represent fruit related expression and downregulated prior to onset of ripening (Jolie et al., 2010). The activities of pectinesterase in this experiment were relatively the same in all of the lines in 3dpb and ripe stages (Figure 5-3). It is shown that the main increase of these enzymes happen at MG stage and in 3dpb and ripe stage remain at its highest level (Hobson, 1963; Rugkong et al., 2010), which correlate with the constant level of enzyme at these stages in Control, CrtZW and CrtZW.DET1. It is shown that polygalacturonase is not well correlated with initial tomato softening (Besford and Hobson, 1972). Extended ethylene treatment was required for induction of PG expression in MG fruits (Lincoln and Fischer, 1988; Yen et al., 1995) although in some studies on tomato mutants such as *RIN*, it is proposed that PG transcripts are either ethylene independent or ethylene is needed for PG mRNA translation (Knapp et al., 1989; Theologis et al., 1993). It is shown that expression of PG gene is around 80% at ripe stage and just around 20% at breaker stage (Seymour et al., 2013a). In this study the PG activity, in correlation with previous studies (Gross and Wallner, 1979; Crookes and Grierson, 1983; Rugkong et al., 2010), represented a very low activity at 3dpb but increased considerably at ripe stage. In CtrZW and CrtZW.DET1 the PG activity increased approximately 3 and 6 times to control respectively (Figure 5-4).

In addition to 2 fold increase in ethylene peak in CrtZW.DET1 line, the PG and β-galactosidases enzymes increases are in line with some other alteration in cell wall

components. Higher amount of AIS and glucuronic acid were the other two cell wall components, which increased in CrtZW.DET1.

In respect to stage of ripening the level of AIS and uronic acid (representing insoluble pectin) decreased from MG to ripe stage, which was expected (Table 5-1 and Table 5-2), and as reported before while the process of ripening going further the amount of insoluble cell wall compounds degrades and solubilise (Janoria, 1974; Campbell et al., 1990; Koch and Nevins, 1990).

The CrtZW.DET1 glucuronic acid ratio to CrtZW represented nearly 3 fold increase whereas to control line showed approximately two fold increase (Table 5-3); this higher difference to CrtZW could confirms the hypothesis of cellular deficiency in CrtZW line (as explained in section 4.7.1.1), which cellular deficiency also reported in other ABA deficient mutant (*hp3*) (Galpaz et al., 2008). Absence of significant difference in the CrtZW.DET1 glucuronic acid ratio to DET1 indicates similarity of this compound in both lines (Table 5-3). Apparently *DET1* downregulated gene compensates the cellular deficiency in CrtZW.DET1 line.

Considering no observation about fruit size changes of CrtZW.DET1 in the second generation to other derived line along with ethylene hormone elevation, enzymes increases (PG and β-galactosidases), cell wall component growth and finally later ripening to CrtZW, it can be proposed that there is an structural improvement or alteration in the cell wall of CrtZW.DET1 which needs two peaks of ethylene and significantly higher amount of enzymes to degrade the cell wall.

In parallel to cell wall alterations as the cell wall disassembled, the susceptibility to different pathogens increase (Prusky, 1996; Flors et al., 2007; Cantu et al., 2008a), but in CrtZW.DET1 with the late ripening and softening characteristics, it is expected to be stronger against pathogens which needs more experimental supports.

Previously the important role of cuticle in ripened was shown by *delayed fruit deterioration (DFD)* with a considerable resistant to postharvest desiccation and stayed firm for a long time (Saladié et al., 2007). Because of the importance of this property, the cuticle thickness of CrtZW.DET1 measured and compared to control which indicate the improved shelf life properties of this line are likely not to be related to changes in cuticle thickness (Figure 5-6).

### 5.3.3 Antioxidant increase also helps shelf life improvement as the second factor

In high anthocyanin purple tomatoes (Zhang et al., 2013) the delay in overripening and enhanced pathogen improved pathogen resistant have been demonstrated and are due to antioxidant improvements in this line which enhanced scavenging ability to decrease levels of reactive oxygen species (ROS). Reduction in tissue damaging by antioxidant activity result the delay in overripening (Zhang et al., 2013). Higher hydrophilic antioxidant/lower ROS levels also affected two cell wall softening genes of *SlPG2a* (polygalacturonase) and *SlTBG4* (β-galactosidase), which represent significantly lower expression in purple fruit during ripening (Zhang et al., 2013). It is proposed that ROS signalling play an important role in the ripening rate, late in fruit development and may delay the processes of overripening (Zhang et al., 2013).

In the DET1 parental line of CrtZW.DET1 also increased total antioxidant content in both polar and nonpolar cellular extracts was reported, which confer the simultaneous elevation of different antioxidants originating from diverse biochemical pathways. It is suggested that core intermediary processes and cellular stress responses is the mechanism underlying this enhancement (Enfissi et al., 2010). In this study for the first time the considerable increase of DET1 shelf life and delay in its overripening have been demonstrated. DET1 line represent 129% and 43% less weight loss and firmness loss by time respectively (see Figure 5-7, Figure 5-8 and Table 5-4). Some other studies demonstrated increased antioxidant capacity or decreased ROS level by different antioxidants could extend the shelf life (Meli et al., 2010; Zidenga et al., 2012). It can be concluded that this delay in overripening and extended shelf life in DET1 is likely to be in due of its overall antioxidants enhancement.

In ZW.DET1 line improvement in the antioxidant level can be considered in two parts. The first phase starts from MG stage till ripening and the second stage covers the over ripening period, which directly linked to the shelf life. In the first part many endogenous antioxidant such as carotenoids (2.4 fold increase to control) and α-tocopherol improved (2.8 times increase to control) similar to DET1 parental line and also non-endogenous ketocarotenoids enhanced (2.6 fold) in comparison to CrtZW parental line (see section 4.2.4.1). The compounds with antioxidant properties are also increased in overripening stage; it is a striking observation that just in CrtZW.DET1 line, 3 groups of antioxidants represent a growing rate in overripening time. Lycopene as the most important carotenoids in tomato, ketocarotenoids and α-tocopherol

indicated 1.3, 1.6 and 2 times improvement during shelf life storage (demonstrated in Figure 5-9). CrtZW also shown active production of ketocarotenoids and lycopene but not tocopherols (see Figure 5-9). This active production of antioxidants in overripenig stage prevents ROS and defending cellular senescence. In CrtZW.DET1 and CrtZW these improvements in variety of antioxidants before ripening and after ripening could count as the second important factor for shelf life improvement in addition to late ABA peak and plausible improved cell wall composition (see Figure 5-11).

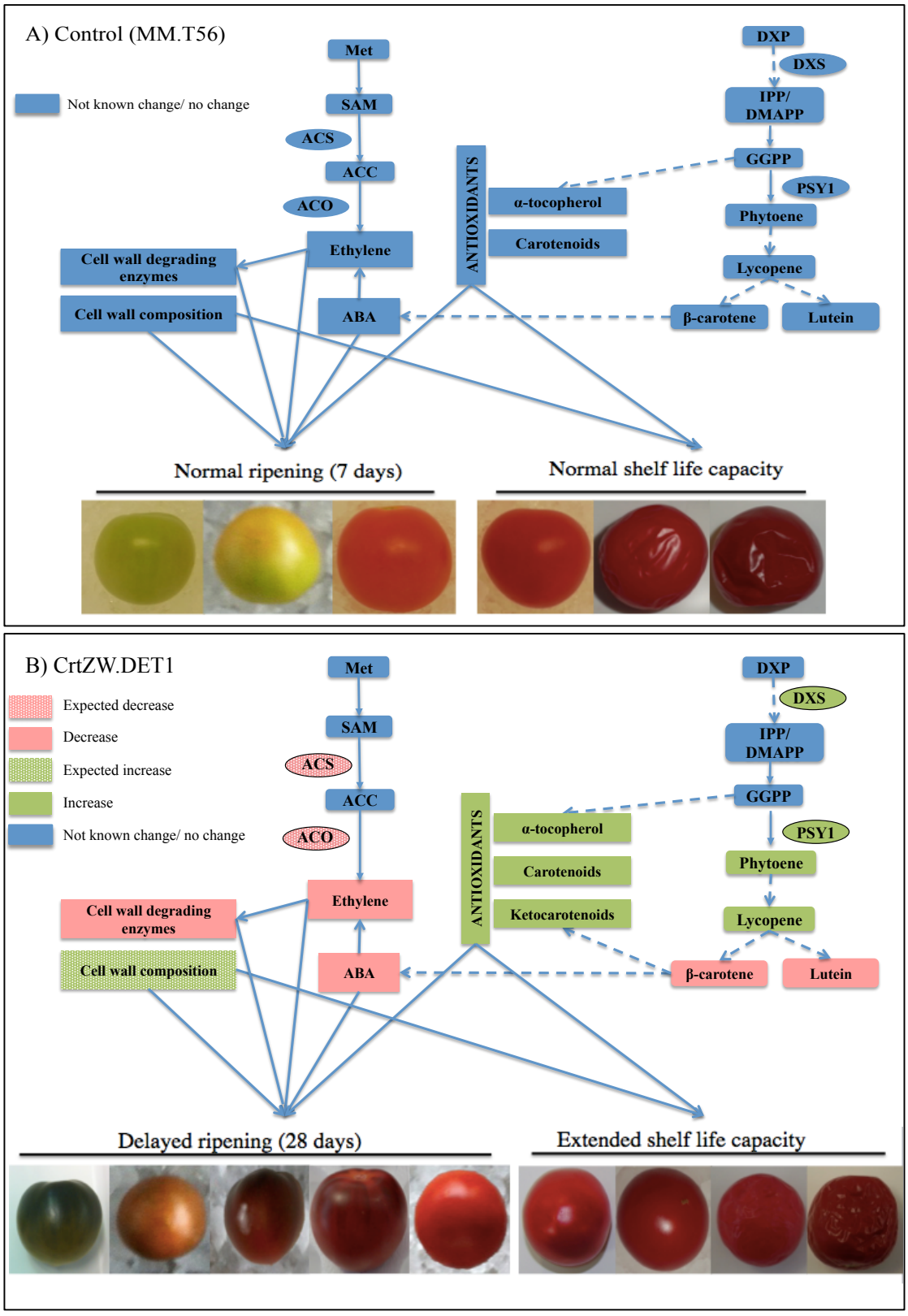



Figure 5-11 The factors effect on ripening and shelf life in Control (MM.T56) line (A); the delayed ripening and extended shelf life mechanism in CrtZW.DET1 line (B).

Data are shown based on detected metabolites, hormones and enzymes levels representing their alteration in Control with normal ripening and CrtZW.DET1 delayed ripening line (see sections

4.2.4.1, 4.2.5, 4.4.1, 5.2.1.1, 5.2.1.2 and 5.2.2 ). Dashed arrows indicate that there are some intermediate compounds between two compounds while solid arrows indicate the direct relations. DXP: 1-deoxy-D-xylulose 5-phosphate, IPP, isopentenyl pyrophosphate; DMAPP, demethylallyl diphosphate; GGPP, gerenaylgeranyl diphosphate; *DXS*, 1-deoxy-D-xylulose-5-phosphate synthase gene; *PSY-1*, phytoene synthase-1 gene; Met, methionine; SAM, *S*-adenosyl methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; *ACS*, ACC synthase gene, *ACO*, ACC oxidase gene; ABA, abscisic acid. Expected increase in ACO and ACC is based on the fact that ABA induce ethylene production by regulation of ACS and ACO gene expression (Zhang et al., 2009).

# CHAPTER 6: GENERAL DISCUSSION

## 6.1 Summary

The overarching aim of this study was the evaluation of different genetic intervention approaches for the enhancement of carotenoids, the effect on antioxidant content and how ABA level could play a role in shelf life extension of *Solanum lycopersicum* fruit.

The first objective related to the identification and validation of putative transcription factors. This included:

A- The *APRR2-Like* transcription factor on chromosome 6. Primary identification on *APRR2-Like* was initiated but needs more investigations.

B- Solyc06g053960 TF designated as *ESB9*; First generation ESB9 plants characterisation demonstrate the effect of this gene on decrease of β-carotene level in all of the lines.

C- Putative candidate genes in the *S.pennelli* sub ILs of 2-3 and 2-4 (Q1968 line) were investigated. The sub-IL Q1968 line which is derived from ILs 2-3 and 2-4 indicated a significant increase specifically in lycopene and also some other metabolites. In the first step RNAseq and metabolomics data have considered and based on the altered transcripts in the introgressed region some candidate genes have been chosen and focused. For the responsible candidate genes some probable underlying mechanism was proposed (see section 3.3.1).

In the second objective non-endogenous ketocarotenoids in tomato have been increased by combining biosynthetic optimisation (astaxanthin biosynthesis of CrtZW line) and transcriptional and regulatory effective genes (*APRR2* and *DET1*). Different strategies were evaluated for the enhancement and production of high-value carotenoid and ketocarotenoid in tomato fruit. The mechanisms underlying the metabolic phenotype and associated physiological changes were investigated. Although in the CrtZW.APRR2 line no dramatic increase was found., but further investigation to elucidate the biochemical molecular mechanisms underlying the CrtZW.APRR2 phenotype provided a deep insight to choosing the best effective candidates for improving high-value compounds (see section 6.1.2.3 and Figure 6-2 for more explanation). On the contrary to CrtZW.APRR2, CrtZW.DET1 exhibited a significant increase in carotenoids both carotenoids such as lycopene and high value ketocarotenoids and also other valuable metabolites such as tocopherols and amino acids.

In the third objective another characteristic of CrtZW.DET1 genotypes were investigated which was delayed ripening display and the hypothesis proposed involved the modulation of abscisic acid (ABA) content in the fruit due to the depletion of β ring derived carotenoids. This direction of research also highlighted associated changes in antioxidant level, cell wall degradation enzyme activities, hormones level and cell wall compound composition. These data have revealed a probable new mechanism for improving the shelf life characteristics of fleshy fruits. Fruit firmness and fruit weight alterations trend in shelf life experiments of CrtZW.DET1 line demonstrated a value added property to this line relating to for its unique shelf life properties and its potential for improving quality in other crops.

As different molecular and genetic manipulation methods were used in this study for improvement of high value compounds, in this chapter the Marker assisted selection method of QTL and gene manipulation breeding strategies from modern breeding techniques (see section 1.7.2) will be explained and the rationale of the strategies used in this study, their importance and their future prospects will be discussed in the appropriate sections.

### 6.1.1 The modern breeding methods used in this studyMarker assisted selection method of Quantitative trait locus (QTL)

The statistical method of QTL links tries to explain the genetic basis of variation in complex traits by linking the phenotypic data to genotypic data (mainly molecular markers) (Falconer and Mackay, 1996; Lynch and Walsh, 1998). This method links the diverse observed phenotypes in population to specific regions of chromosomes. A variety of QTLs such as yield, metabolism, morphology, plant adaptation and etc. have been applied in tomato fruits for many years, which has resulted in production of numerous introgression lines (ILs) populations. Each introgressed line carries a single chromosome segment from one of its parental wild species (Lippman et al., 2007; Eshed and Zamir, 1995). For instance fifty ILs introduced from *Lycopersicon pennellii* (green fruit) and the *M82* line. Each of the lines includes a single homozygous segment of *L. pennellii* chromosome. All of 50 ILs together provide complete coverage of the genome (Eshed and Zamir, 1995).

In this study a subIL 2-3 and 2-4 of *L. pennellii* and the M82 was characterised. This sub IL in different generation represents high lycopene content. The levels of lycopene represent 2-fold increase to M82 wild type.

This improvement in lycopene makes this sub IL a reasonable candidate for improved consumer traits without GM related concerns. Although a single responsible gene could not identified for high lycopene phenotype, a number of candidate genes constituting a complex trait are proposed for the overall changes in some of important biochemical processes. These candidates and the resulting material require further investigations (see section 3.3 and 6.2).

### 6.1.2 Gene manipulation of high-value compounds and Ripening-Related Genes

Genetic engineering is the manipulation of the genetic material present in the organism. Gene manipulation nowadays use vastly for different means such as enhancing the accumulation of endogenous metabolites, productions of new metabolites via introduction of new genes beyond their natural genetic pool, studying the unknown gene functions and mediating the degradation of compounds are some of the targets in genetic engineering. Manipulation of organisms can involve the silencing, down regulating or upregulating the desired genes. Gene silencing is the principal method in understanding and analysing unknown gene functions. Gene silencing consist of antisense gene silencing and RNAi gene silencing methods. The antisense RNA, is a strand of nucleotides which binds to the mRNA to block the production of the protein based on its complementary property (Hamilton and Baulcombe, 1999). The second method of RNA interference (RNAi) also known as co-suppression is post-transcriptional gene silencing and quelling. In this method the introduced synthetic double strand RNA robustly supress the expression of desired endogenous gene. The performance of this method is better than antisense RNA and can be used vastly in large-scale gene identifications (Kupferschmidt, 2013). The silenced ESBs transgenic lines, which are important TFs derived from neural network analysis, have been produced by RNAi technology.

These different gene modification methods can be used under various engineering strategies. Figure 6-1 illustrate the direction that could be considered in gene manipulations. The pathway of desired metabolites could be targeted via different strategies for instance, improved transcription (Figure 6-1 part 2); enhanced translation (Figure 6-1 part 3); improved transcription and translation (Figure 6-1 part 4). The precursors pathway could be targeted. Enhancing transcription via alteration in transcription factors expression effecting the regulation of the desired pathway

(Figure 6-1 part 5), plastid transformation (Figure 6-1 part 6), elimination of divided pathways that consume the desired metabolites or degrade it (Figure 6-1 part 7, 9, 13 & 14) and enhancing the transporters/sequestration compartments (Figure 6-1 part 11& 12) also could be considered as other strategies for optimising a metabolite production. It is also possible a new shortcut pathway introduce to organism for production of desired metabolite with heterologous enzymes (Figure 6-1 part 1). Interaction with intermediary metabolism and other plant processes (Figure 6-1 part 8) and metabolites plastidial modification (Figure 6-1 part 10) are other strategies for this means.

For instance, in tomato fruits many studies have been performed to study the regulation of light signal transduction components, elevation the high value compounds such as antioxidants, pigments for example anthocyanin, carotenoids and ketocarotenoids, alteration in cell wall components and enzymes and also phytohormones for extending the shelf life of fruits. Alteration in transcription factors expression effecting the regulation of the desired pathway (Figure 6-1 part 5) and combination of the effect of TFs with constructed new pathway with heterologous enzymes were used in objective 1 and 2 (see section 1.8) of this study. The more detailed examples of these targets are discussed in the following three sections.

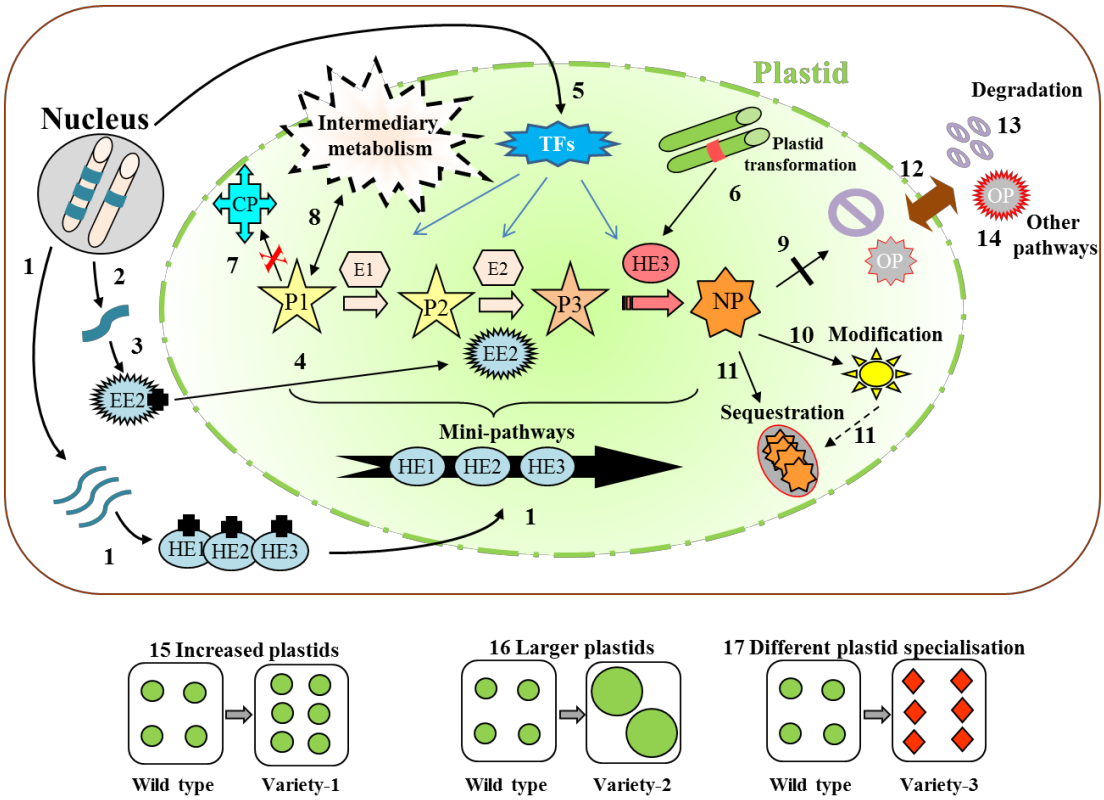



Figure 6-1 Schematic presentation of potential strategies for improvement of secondary metabolites by metabolite engendering (adapted from Fraser, personal communication, Nogueira, 2013)

"P, product; E, enzyme; HE, heterologous enzyme; EE, enhanced enzyme (transcriptionally or/and translationally); NP, new product; OP; other products derived from the new product; CP, competing pathway; TFs, transcription factors. 1, Construction of a mini-pathway with heterologous enzymes; 2, improved transcription; 3, enhanced translation; 4, improved transcription and translation; 5 enhanced transcription via transcription factor expression; 6, plastid transformation; 7, restriction of the diversion of precursors; 8, interaction with intermediary metabolism and other plant processes; 9, minimizing non-enzymatic degradation and the diversion of new products into other pathways; 10, metabolites plastidial modification; 11, sequestration of metabolites; 12, manipulating transport mechanisms; 13; controlling the rate of non-enzymatic degradation; 14, controlling the rate of diversion into other catabolic pathways."

### 6.1.2.1 Regulatory genes manipulation

Manipulation of regulatory genes has been used for the improvement of desired metabolites. In tomato fruits many attempts have been reported, that have resulted in the improvement of endogenous carotenoids or the introduction of new pigments to fruit such as ketocarotenoids and anthocyanin. Extending the shelf life capacity of this crop also has been targeted for many years via alteration in cell wall degradation components genes or manipulation of phytohormones related gene transcript. These aspects are explained in the two next sections.

#### 6.1.2.1.1 Pigment Development

Many of carotenoid pathway gene products manipulated for improving the carotenoid levels in tomato fruits. For instance in an early attempt in 1991 the *PSY* (*TOM5*) expression significantly reduced in TOM5 antisense tomato, resulted yellow tomato fruit and pale yellow flowers (Bird et al., 1991). Bacterial phytoene synthase (*CrtB*) was overexpressed in tomato fruits under PG promoter resulted an increase of 1.8 fold in lycopene content and 5 fold in β-carotene (Fraser et al., 2002). An overexpressed *DXS* gene originated from *E.coli* resulted in 1.6 fold increase in total carotenoid level of tomato fruit (Enfissi et al., 2005). Four fold increase in β-carotene level reported from an overexpressed citrus *LYCB* in tomato fruits (Guo et al., 2012). The Table 6-1 represent other attempts for improvement of carotenoids via manipulation of pathway genes; all of the colours except grey and dark blue indicate different strategies for manipulation of carotenoid biosynthesis pathway genes.

Introduction of other pigments to tomato plant beyond their natural carotenoids was a concern over past years. Ketocarotenoids as a high value compound with considerable antioxidant and colorant properties used extensively in animal feeds (such as chicken) and in aquaculture like salmon and shrimp (see section 1.5.1.1.1). Existence of carotenoid pathway and active production of carotenoids in tomato plant make it a good host for production of ketocarotenoids from this pathway. *CrtZ* (β-carotene hydroxylase) and *CrtW* (β-carotene ketolase) genes were introduced to tomato in different attempts. In the first attempted transgenic tomato expressing the *CrtW* and *CrtZ* from *Paracoccus sp*. Just represent very small amounts of ketocarotenoids in tomato leaf and fruit (Ralley et al., 2004).

It is suggested that this negligible amount of ketocarotenoids is in due of poor cooperation of tomato carotenogenic enzymes with bacterial transgene products (Huang et al., 2013). In a successful research CrtZ gene from *Haematococcus pluvialis* and algal *CrtW* gene from *Chlamydomonas reinhardtii* transformed under CaMV 35S promoter in two tomato variety of wild type (WT) (accumulating mainly lycopene) and a mutant of B-type (BT) that synthesizes mainly β-carotene and minor amount of lycopene. It is interesting to note that the robust increase in astaxanthin level were reported in transformed BT but not in the wild type tomato (Huang et al., 2013). Transformed wild type tomatoes containing both of *CrtZ* and *CrtW* genes (WT-bktbhy20) only produce low amount of ketocarotenoids 20.8 μg/g DW. The total endogenous carotenoids improved 1.9 fold to their wild type untransformed plants

(data was extracted from Huang et al., 2013). BT line containing both of transformed genes (BT-bktbhy8) result in production of 3.12 mg/g free astaxanthin in leaves and up to 16.1 mg/g esterified astaxanthin, which is prominent result (Huang et al., 2013).
In an unpublished attempt the bacterial genes of *CrtZ* (*β-carotene hydroxylase*) and *CrtW* (*β-carotene ketolase*) genes were originated from a marine bacteria *Brevundimonas sp* strain SD212 with specific plant codon (Hasunuma et al., 2008) introduced to wild type Money maker tomato fruit (Enfissi et al., unpublished). The transformed tomato (CrtZW) in this method produced approximately 100 μg/g DW that is 4.8 fold higher in comparison to WT-bktbhy20. The levels of endogenous carotenoids also show an improved level (1.5 fold) compared to the control line. The CrtZW line represents the best result on wild type tomato engineered plants for production of ketocarotenoids before the production of CrtZW.DET1 line (Enfissi et al., unpublished). In this study this line used as one of the parents for genetic cross of CrtZW.DET1 line and shown as CrtZW. The results discussed in the present study have resulted from the use of the CrtZW plants as parental lines, which may have some different with original data due to environmental alterations such as summer or winter crops (Enfissi et al., unpublished).
The alteration of some of non-carotenogenic genes, which result in improved carotenoid level, is another strategy that shown in Table 6-1 with grey and dark blue colours. Light transduction component or lipid associated proteins (enhance sequestration capacity) are the main targets of this strategy. Downregulated light transduction DET1 gene with fruit specific promoters represent 6 fold increase in total carotenoid levels (Enfissi et al., 2010). In this study the inheritable potential of DET1 line for enhancing carotenoid production used for improvement of nonendogenous ketocarotenoids via genetic cross between CrtZW and DET1 genotypes.
The improvement of high value compound by focusing on regulatory genes represents considerably successful result in CrtZW.DET1 line. The level of total ketocarotenoids in this line enhanced 2.6 and 13 fold to CrtZW (Enfissi et al., unpublished) and WT-bktbhy20 (Huang et al., 2013) respectively. The endogenous carotenoids also improved 2.4 times to control line.
Other improved aspects of CrtZW.DET1 which consist of extended shelf life, improved antioxidants, alteration in cell wall and phytohormones production will be discussed in the next section.

Table 6-1 Different engendering strategies resulted in carotenoids and ketocarotenoids enhancement in *Solanum lycopersicum* (selected from Nogueira, 2013; Farre et al., 2010).

Different colours represent various engineering strategy. "Light pink, tissue-specific overexpression of one carotenogenic gene; light yellow, overexpression of one carotenogenic gene; green, overexpression of several carotenogenic genes; blue, RNAi of carotenogenic gene; dark blue, RNAi of non-carotenogenic gene; grey, overexpression of non-carotenogenic genes; yellow, plastid transformation." Highlighted cells correspond to the higher levels of carotenoids and ketocarotenoids produced in tomato .

| Species | Genes (origin) | Function | Promoters | Carotenoid levels in transgenic plants | Engineering strategy | References |
|---|---|---|---|---|---|---|
| Tomato (*Solanum lycopersicum*) | *Dxs* (*Escherichia coli*) | DXP synthase | Fibrillin (pepper) | 7200 µg/g DW total carotenoid in fruit (1.6-fold)<br>700 µg/g DW β-carotene (1.5-fold); 6700 µg/g DW lycopene (1.6-fold) | tissue-specific overexpression of one carotenogenic gene | Enfissi et al., 2005 |
| | *Psy-1* (tomato) | phytoene synthase | CaMV35S | 3615 µg/g DW total carotenoid in vegetative tissue (1.14-fold)<br>detection of lycopene (386 µg/g DW) and small increase of β-carotene | overexpression of one carotenogenic gene | Fray et al., 1995 |
| | *Psy-1* (tomato) | phytoene synthase | CaMV35S | 2276.7 µg/g DW total carotenoid in ripe fruit (1.25-fold)<br>819 µg/g DW β-carotene in ripe fruit (1.4-fold) | overexpression of one carotenogenic gene | Fraser et al., 2007 |
| | *Crt* B (*P. ananatis*) | phytoene synthase | Polygalacturonase (fruit specific) | 5918 µg/g DW total carotenoid in ripe fruit (2-fold)<br>825 µg/g DW β-carotene (5-fold); 56 µg/g DW phytoene (1.6-fold); 5137 µg/g DW lycopene (1.8-fold) | tissue-specific overexpression of one carotenogenic gene | Fraser et al., 2002 |
| | *Crt* I (*P. ananatis*) | lycopene desaturase | CaMV35S | 520 µg/g DW (1.9-fold) β-carotene in fruit<br>reduced lycopene and phytoene content | overexpression of one carotenogenic gene | Romer et al., 2000 |
| | *Lyc-b* (*Arabidopsis*) *Chy-b* (*Capsicum annuum*) | lycopene cyclase β-carotene hydroxylase | pds (fruit specific; tomato) | 63 µg/g FW β-carotene in fruit (12-fold)<br>β-cryptoxanthin (11µg/g FW) and zeaxanthin (13 µg/g FW) were produced | tissue-specific overexpression of one carotenogenic gene | Dharmapuri et al., 2002 |
| | *Lyc-b* (*Arabidopsis thaliana*) | lycopene cyclase | pds (fruit specific; tomato) | 57 µg/g FW β-carotene in fruit (7-fold); no affect on lycopene | tissue-specific overexpression of one carotenogenic gene | Rosati et al., 2000 |

| Species | Genes (origin) | Function | Promoters | Carotenoid levels in transgenic plants | Engineering strategy | References |
|---|---|---|---|---|---|---|
| Tomato (*Solanum lycopersicum*) | *Lyc-b* (antisense; *Arabidopsis thaliana*) | lycopene cyclase | pds (fruit specific; tomato) | 85 µg/g FW lycopene in fruit (1.6-fold) | RNAi of carotenogenic gene | Rosati et al., 2000 |
| | *Lyc-b* (tomato) | lycopene cyclase | CaMV35S | 215.2 µg/g FW total carotenoid in fruit (2.3-fold) | overexpression of one carotenogenic gene | D'Ambrosio et al., 2004 |
| | *Lyc-b* (citrus) | lycopene cyclase | CaMV35S | 205 µg/g FW β-carotene in fruit (46-fold); almost no lycopene left<br>1105 µg/g DW β-carotene in fruit (4-fold)<br>30% increase of total carotenoid in fruit | overexpression of one carotenogenic gene | Guo et al., 2012 |
| | *Lyc-b* (daffodil) | lycopene cyclase | Ribosomal RNA | 950 µg/g DW β-carotene (4.5-fold) in fruit | plastid transformation (overexpression of one carotenogenic gene) | Apel and Bock, 2009 |
| | *Cyc-b* (tomato) | lycopene cyclase | CaMV35S | increased % of β-carotene (6-fold) | overexpression of one carotenogenic gene | Ronen et al., 2000 |
| | *Cyc-b* (antisense; tomato) | lycopene cyclase | CaMV35S | increased % of lycopene (1.1-fold) | RNAi of carotenogenic gene | |
| | *Crt*Y (*P. ananatis*) | lycopene cyclase | aptI | 286 µg/g DW β-carotene in fruit (4-fold) | tissue-specific overexpression of one carotenogenic gene | Wurbs et al., 2007 |
| | *SlNCED1* | 9-*cis*-epoxycarotenoid dioxygenase | E8 fruit-specific | 210 µg/g FW lycopene in pulp (1.5-fold); 40 µg/g β-carotene (2.4-fold)<br>275 µg/g FW total carotenoid in pulp (1.6-fold) | RNAi of carotenogenic gene | Sun et al., 2012 |
| | *CrtR*-b2 (tomato) | β-carotene hydroxylase | CaMV35S | 1589 µg/g DW violaxanthin in leaf (3.5-fold)<br>3453 µg/g DW total carotenoid in leaf (3.5-fold) | overexpression of one carotenogenic gene | Giorio et al., 2012 |
| | *Crt*W and *Crt*Z (*Paracoccus sp.*) | β-carotene ketolase and β-carotene hydroxylase | CaMV35S | Ketocarotenoid formation in leaves, low levels detected in the fruit | overexpression of several carotenogenic genes | Ralley et al., 2004 |
| | *Bkt* (*Chlamydomonas reinhardtii*) and *Bhy* (*Haematococcus pluvialis*) | β-carotene ketolase and β-carotene hydroxylase | CaMV35S | 5050 µg/g DW total ketocarotenoid in leaves | overexpression of several carotenogenic genes | Huang et al., 2013 |

| Species | Genes (origin) | Function | Promoters | Carotenoid levels in transgenic plants | Engineering strategy | References |
|---|---|---|---|---|---|---|
| Tomato (*Solanum lycopersicum*) | Chrd (cucumber; antisense) | lipid associated protein | CaMV35S | Reduced carotenoid levels in flower | overexpression of non-carotenogenic genes | Leitner-Dagan et al., 2006 |
| | Fibrillin (*Capsicum annuum*) | lipid associated protein | Fibrillin (pepper) | 150 µg/g FW β-carotene (1.5-fold); 450 µg/g FW lycopene (2-fold); increased derived volatiles in fruit | overexpression of non-carotenogenic genes | Simkin et al., 2007 |
| | *Cry2* (tomato) | blue light photoreceptors | CaMV35S | 1490 µg/g DW total carotenoid ripe fruit pericarps (1.7-fold) 101 µg/g DW β-carotene ripe fruit pericarps (1.3-fold) | overexpression of non-carotenogenic genes | Giliberto et al., 2005 |
| | *Det-1* (tomato, antisense) | light signal transduction | P119, 2A11 and TFM7 (ripening enhanced) | increased β-carotene (8.5-fold) and lycopene (2.2-fold) and increased flavonoid content in red-ripe fruit | RNAi of non-carotenogenic gene | Davuluri et al., 2005 |
| | *Det-1* (tomato, antisense) | light signal transduction | P119, 2A11 and TFM7 (ripening enhanced) | 1455 µg/g DW β-carotene (15-fold); 1574 µg/g DW lycopene (5-fold); 4179 mg/g DW total carotenoid (6-fold) in ripe fruit | RNAi of non-carotenogenic gene | Enfissi et al., 2010 |
| | *Cul4* | light signal transduction, DDB1 interacting protein | TMF7 (ripening enhanced) | 59 µg/g FW β-carotene (2-fold); 310 µg/g FW lycopene (2-fold) in fruit | overexpression of non-carotenogenic genes | Wang et al., 2008 |

#### 6.1.2.1.2 Shelf life extension

One of the main concerns for the tomato industry is post harvest resistance and shelf life capacity. Post harvest losses result huge economic losses every year. Different strategies have been employed for tomato shelf life extension. Harvesting mature green tomatoes and induction of ripening by ethylene gas was one of the first strategies for this means. But loss of flavour and lack of desired taste was a big problem of this conventional method (Maul et al., 1998, 2000).

Application of ethylene signalling inhibitors also used for this goal. Exogenous treating of tomatoes with an ethylene inhibitor with 1-MCP (1-methyl- cyclopropane, an ethylene-action inhibitor) in different stages of ripening (MG, breaker, orange, and red) represent decrease in transcription of *ACOI, PSY1* and *EXPI* (Hoeberichts et al., 2002) and also treatment of fruits after breaker with 1-MCP result in extension of shelf life without noticeable flavour loss. But this method is a costly method and is not applicable in large scales (Baldwin et al., 2011). Using the natural ripening mutants was another strategy for preventing the post harvest losses. Crossing of ripening mutants with commercial varieties result is heterozygous hybrids for ripening mutations which could considerably extend the shelf life (Kopeliovitch et al., 1979). Heterologous form of some of mutants were introduced to market such as NOR (nonripening), NR (never-ripe), RIN (ripening inhibitor), and alc (Alcobaca) (Mutschler et al., 1992). The third strategy, which is the main concern of this section, is genetically modified crops for means of shelf life extension. Genetic manipulation of ethylene hormone genes, cell wall degrading component genes and improving the antioxidants and polyamines are the main used techniques.

##### 6.1.2.1.2.1 Reduction of ethylene

In respect to important effect of ethylene signalling role in fruit ripening, manipulation of the relative genes counted as effective method for delay the ripening and shelf life improvement.

Introduction of antisense ACC oxidase (*ACO*) gene in tomato fruits decreased the ethylene level considerably (Hamilton et al., 1990). Transgenic antisense ACS tomato represents a considerable ethylene production inhibition (99%), which never fully ripened unless treated with exogenous ethylene (Oeller et al., 1991). An over expressed ACC deaminase (the bacterial enzyme using ACC as a substrate) transgenic tomato represent significantly firmer fruits than controls while the ethylene synthesis

was inhibited to some extend and ACO and PG activity showed no significant differences to control. This data suggest that other enzyme play important roles in fruit softening (Klee et al., 1991; Klee, 1993). In another attempt a new gene of S-adenosylmethionine (SAM) hydrolase (*E.coli* origin) introduced to tomato. This enzyme with methylation of SAM, which is the precursor of ACC cause reduction of this compound and consequently reduced production of ethylene (Good et al., 1994).
This study demonstrates a new strategy for induction of delay in ripening. In CrtZW and CrtZW.DET1 active production of a new introduced side chain to carotenoids pathway (heterologous ketocarotenoids biosynthesis pathway) reduced the level of ABA precursor and consequently delayed the peak of ABA at first and then ethylene production (see section 5.3.2). In this method production of high value ketocarotenoids result in a value added to this line in comparison with inhibitory methods, which directly manipulate ethylene biosynthesis pathway as explained above. In other words, instead of stopping the process of hormone accumulation, by introducing a new side pathway for consumption of hormonal precursors, guide the pathway for production of other valuable compounds (Ketocarotenoids) rather than stopping the process. With this strategy an 18 days delay in ripening improved the shelf life of this crop and delayed the softening considerably. Also considering the natural peak of ABA and ethylene (but just with a delay) in this line result in normal flavour and taste of this crop in comparison to exogenous ethylene treatment strategy. The improved antioxidant property of this line will be discussed in its section. In conclusion it is important to consider the multiple ability of some regulatory genes for improvements of crops which some times have been neglected.

6.1.2.1.2.2 Cell Wall enzyme manipulation

Suppressing the genes of cell well degradation enzymes have been good targets for improving the shelf life and delaying softening.
As it was believed that PG is a key enzyme in softening of tomato fruit, PG was the manipulated gene for postponing the softening. The antisense mRNA method used for suppression of its expression that result in decrease of the transcripts to just 1% of wild type tomatoes. But surprisingly with reduction of PG activity and inhibition of pectin degradation, the process of softening was not different to wild type (Smith et al., 1988). Also overexpressed PG gene in RIN mutant could not restore the normal softening (Giovannoni et al., 1989). The same observation for PME also reported.

Fruit softening of antisense PME transgenic fruits did not present a difference with control (Tieman et al., 1992) but interestingly an antisense RabII GTPase gene decreased PME and PG level also induced reduction of softening which suggesting the role of RabII GTPase gene in secretion or transportation of cell wall degrading enzymes (Lu et al., 2001). A delayed ripening tomato produced by gene silencing of two N-glycoprotein modifying enzymes: α-mannosidase and β-D-N-acetylhexosaminidase. These transgenic tomatoes last for 45 days in room temperature without rotting while normal tomatoes last for maximum 15 days (Meli et al., 2010). EXP1 overexpressed fruits represent softer fruits and downregulated ones were firmer through the ripening (Brummell et al., 1999b). Also the antisense TBG4 (β-galactosidase/exo-galactanase) fruits resulted 40% firmer fruits to control (Smith et al., 2002).

6.1.2.1.2.3 Antioxidants and polyamines

In overall silencing the ripening inducers or overexpression of ripening inhibitors could extend the shelf life of tomato fruits (Centeno et al., 2011). In tomato fruit introduction of two TFs of *Del* and *Ros1* from snapdragon *Antirrhinum maju* result in significant production of anthocyanin with purple phenotype. These purple tomatoes represent doubled shelf life property in result of production of high levels of anthocyanin antioxidants (Zhang et al., 2013). The high production of antioxidant level in DET1 have been reported in result of downregulated *DET1* gene (Enfissi et al., 2010). The shelf life property of this genotype investigated in this study, which represent considerable improvement in shelf life in result of 129% and 43% less weight loss and firmness loss by time. This improvement could be in due of activity of variety of antioxidants against cellular oxidants as antioxidant improvement was one of the main criterion of DET1 characteristics (Enfissi et al., 2010). In CrtZW.DET1 also the level of various antioxidants improved which in correlation with delayed ABA and ethylene pack seems to be responsible factors for considerable improvement in shelf life property of this line (of 110% and 47% less weight loss and firmness loss by time). These data indicate that manipulation of high value compounds with antioxidant property could also improve the shelf life property of new transgene as a second target.

Basically the level of polyamines decreased during ripening therefore overexpression of polyamines such as spermidine also used as another strategy for shelf life improvement (Nambeesan et al., 2010).

#### 6.1.2.2 TFs manipulation and their overall effects

An overview about different TFs and their roles in fruit development and ripening were explained in section 1.5.2. Here some studies on manipulation of TFs, which result in metabolite improvement, will be mentioned.

Overexpression of a zinc finger SlZFP2 (play role in ABA biosynthesis during fruit development) caused multiple phenotypic alterations, for instance delayed fruit ripening, more branches, early flowering, faster seed germination and lighter seeds but on the other hand its downregulation resulted in inhibited seed germination, accelerated ripening and problematic fruit set (Weng et al., 2015).

The overexpression of APRR2-Like TF enhanced the levels of plastid numbers, area and chlorophyll content in immature fruits and elevated the carotenoids level in ripe fruits. The overexpression APRR2-Like line also represented upregulation of some other ripening related genes along with *APRR2-Lik* gene alteration which indicate the available link between the expression of *APRR2-Lik* gene and the ripening process (Pan et al., 2013). This high pigment crop was chosen as a parental line for genetic cross between TFs and regulators (CrtZW), which will be explained in the next section.

The production of anthocyanin pigment with considerable antioxidant effects in tomato fruits mediated by introduction of *Del* and *Ros1* TFs from snapdragon to the tomato gene pool (Zhang et al., 2013). The effect of TFs usually is not as direct as observed the direct effect of the two snapdragon TFs on anthocyanin production and cover a vast range of effects on different components. For instance RIN is a master transcription factors which activating many various regulatory genes and also other TFs in fruit ripening. *RIN* mutant do not ripen even with exogenous ethylene application. Over 240 RIN targets were introduced which take part in various functions from effect of RIN on other TFs such as NOR, CNR, SlAP2A, TDR4 and HB-1 to other aspects of ripening for instance pigment accumulation, ethylene biosynthesis and responses, aroma production and cell wall hydrolysis (Martel et al., 2011; Qin et al., 2012; Fujisawa et al., 2013; Kumar et al., 2014). Ten RIN downstream candidates TFs resulted from an artificial network interference analysis

have been silenced by RNAi method for better characterisation as detailed in section 3.2.2. These transgenic lines designated as ESB lines. This study was carried out the characteristics of 10 primary transformed lines of ESB9 (Solyc06g053960). The primary analysis of these lines represent a considerable reduction in pigments and specifically the β-carotene content which suggest the effect of this TF on pigmentation. But needs further studies.

In conclusion considering the usual vast effects of TFs on other cellular factors, manipulation of TFs strategy usually alters different cellular aspects for instance ripening process. Considering the affecting area of the manipulated TFs could join with a regulatory altered gene for enhancing the desired specific metabolite. The more detailed examples of this method explained in the next section.

#### 6.1.2.3 Combination of manipulated TFs and regulatory genes

Considering the mechanism of manipulated gene that how effecting the cellular pathways and predicting the pathway interactions or inhibition of combined genes is a necessary point for designing the combining manipulated regulatory and TFs for producing new genotypes. For clarification three combined examples of TFs with regulatory genes will be discussed.

The first example is the new of CrtZW.APRR2 genotype from genetic cross between regulatory gene of *CrtZW* and overexpressed TF of *APRR2-like*. This combination of genes did not represent a good result for enhancing the ketocarotenoids. As demonstrated in section 4.7.2 that *CrtZW* genes by active consumption of ABA hormone precursor result in delayed peck of ABA and ethylene hormone.

Considering the alteration of important *RIN*, *NOR* and *CNR* TFs in overexpressed *APRR2-Like* and the direct effect of these TFs on ethylene signalling (Pan et al., 2013), it was suggested that *APRR2-Like* effects via this main regulatory genes and ethylene in the second steps via ethylene signalling pathway (Section 4.7.2). The improvement in plastidal parameters and pigment enhancement in CrtZW.APRR2 line was not as impressive as downregulated *APRR2-Like* gene because of *CtrZW* gene indirectly leads to delay in production of ABA and then ethylene production. This means interference with the mechanism of effect of *APRR2-Like* gene, which is via master regulators and ethylene signalling. Therefor downregulated *APRR2-Like* gene could not put its positive effect on pigmentation in the CrtZW.APRR2 genotype (Figure 6-2).

In another study the combination of regulatory genes of *CrtZ* and *CrtW* with putative TFs used as a potential strategy for ketocarotenoids improvement (Nogueira, 2013). Each of *Phytochrome-Interacting Factor 5* (*PIF5*) and *Arabidopsis Response Regulator 14* (*ARR14*) TFs were transformed along with CrtZW gene in tobacco transient system separately for testing the output of transgenic combination. The ZW-PIF5 leaves did not represent higher ketocarotenoids levels to ZW. But ZW-ARR14 represents higher level of ketocarotenoids (1.3 fold) (Nogueira, 2013).

PIF5 known to effect ethylene biosynthesis and phytochrome signalling (Khanna et al., 2007), it is also shown PIF5 to play role in regulation of *PSY* gene expression (Toledo-Ortiz et al.,2010). Considering the absence of ketocarotenoids level in ZW.PIF5 transient tobacco, it could be in result of CrtZW gene which neutralizing the effect of PIF5. It was explained in detailed in section 5.3.2.1 that *CrtZW* genes by active consumption of ABA hormone precursor result in delayed peak of ABA and ethylene hormone. In respect to demonstrated effect of PIF5 on ethylene biosynthesis, this delay in production of ethylene could also delay the effect of PIF5 and its other effects for instance *PSY* gene regulation. It could be suggested that the other effects of PIF5 (phytochrome signalling and regulation of *PSY* gene expression) occur after ethylene production or is dependent to ethylene production (Figure 6-2). PIF5 negative effect on accumulation of pigments and ketocarotenoids in PIF5.CrtZW transient cells is similar to *APRR2-Like* gene in CrtZW.APRR2 line both with effect on ethylene signalling.

The ZW-ARR14 combination was an appropriate combinations of target genes for improvement of ketocarotenoids as ARR14 takes part in regulating the cellular response to cytokinin (Mason et al., 2005) and also potentially in light signalling (D'Agostino and Kieber, 1999). Similar to DET1 gene, which is a light signal transduction gene, ARR14 also is a light signalling gene and puts its effects independently from ethylene production genes and ethylene signalling genes. Therefor these two genes can correlate successfully for improvement of ketocarotenoids without any interference (Figure 6-2). Therefore, it can be suggested that genes with independent effects from ethylene biosynthesis and its signalling pathways via important *RIN, NOR* and *CNR* TFs and other important TFs are potential candidates (see section 6.2) for combination with CrtZW and consequently improvement of ketocarotenoids level (Figure 6-2).

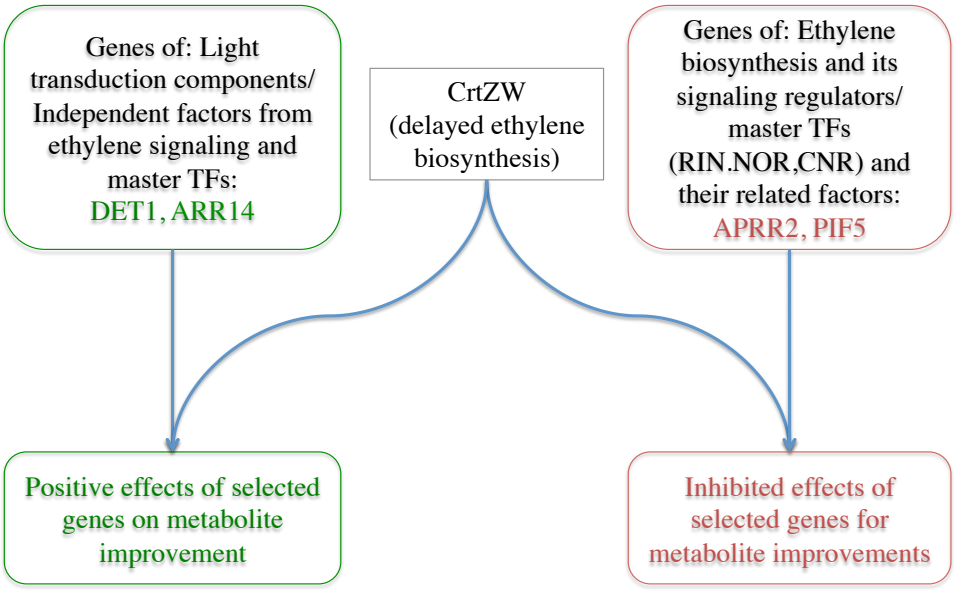



Figure 6-2 Schematic presentation of combination *CrtZW* gene and the effective or inhibitory candidate genes for ketocarotenoids and other metabolites improvement.

## 6.2 Future directions and recommendations

Objective1-A: Finding APRR2-like homologue

As a proper APRR2-like homologue was found on the Sol genomic network (see section 3.2.1) and also considering the wild effect of APRR2-like gene on plastids and pigment contents (Pan et al., 2013), it is suggested to test this homologue in the transient gene expression system. If the primary result was acceptable, then making stable transformed plants and primary characterisation of them can be planned. In that case it is important to continue the experiments in the second generation to have robust data about the transformed plants.

Objective 1-B: Transformed ESB9 plants

The primary characterisation of 10 independent transformed ESB9 lines have performed in this study as described in section 3.2.2. ESB9-2, ESB9-3 and ESB9-8 with a decrease in all of carotenoid contents at ripe stage were selected for more investigation. For the second generation some primary experiments for instance phenotype characterisation and pigment analysis along side with some more advanced experiment for example metabolomics and gene expression can provide good

information about this transformed genotype. If in the second generation rewarding results were gained, making an over expressed transgenic plants of this gene could improve the carotenoid levels.

Objective1-C: Q1968

As this line is not a transgenic line, it can be introduced to the market as a high lycopene line but further experiment could complete the conclusion about this sub IL. These experiments could be consisted of repeating the gene expression for the important TFs underlying this region. This data would help about conclusion about their probable role in Q1968 phenotype. Subsequently the next stage in the process would be making some silenced/over expressed transgenes from the approved TFs and effective functional genes such as cytochrome P450 (Solyc02g082070.2) (section 3.3.1) which not only affect carotenoid pathway but also other biochemical pathways.

Objective 2: Genetic crosses of CrtZW.DET1

The level of total ketocarotenoids in CrtZW.DET1 line enhanced 2.6 fold to CrtZW (Enfissi et al., unpublished) which makes this genotype a valuable candidate for ketocarotenoids production for animal feedstock in industrial scale (For instance salmons and shrimps); but for more improvement in ketocarotenoid contents other strategies could be helpful. Considering the surprising increase of astaxanthin level in the transformed high β-carotene BT-bktbhy8 line (Huang et al., 2013), a bright prospect could be considered for crossing the CrtZW.DET1 line with a high β-carotene lines such as naturally mutated lines like Galápagos tomatoes/*Solanum galapagense* (*Lycopersicon cheesmanii*) (Mackinney et al., 1954), *Lycopersicon esculentum Mill* breeding lines of 97L63, 97L66, and 97L97 (Stommel, 2001) and B-type tomatoes (BT) (Huang et al., 2013) or genetically transformed high β-carotene lines for instance manipulated *LYCB* gene in tomato fruit from different origins: citrus origin, (Guo et al., 2012), tomato origin (D'Ambrosio et al., 2004) , Arabidopsis thaliana (Rosati et al., 2000) and CrtI transgenic line (*P. ananatis* origin) (Römer et al., 2000).

Objective 3: Delayed ripening lines and shelf life improvement

Other improved aspects of CrtZW.DET1 line which affects on extended shelf life property of this line were consisted of firstly, increased antioxidant levels

For the first part which is improved antioxidants levels, it could be interesting to look at the level of antioxidant activity in polar and nonpolar phase separately. There are different compounds with antioxidant effect in ripe tomato; for instance, flavonoids, phenylpropanoids and ascorbic acid (vitamin C) which are active in polar (hydrophilic) phase and also bioactives in nonpolar (hydrophobic) phase for example carotenoids and tocopherols. Measuring the effect of antioxidants in different environments by specific tests such as trolox-equivalent antioxidant capacity (TEAC) analysis could give a good insight about the altered antioxidants activity of CrtZW.DET1 line to the control line.

The second improved aspects of CrtZW.DET1 line, which is delayed ABA and ethylene peaks because of its precursor (β-carotene) consumption in a new introduced pathway (ketocarotenoids production pathway) to this line, can be considered as a new strategy for improving the shelf life of other crops to see whether this strategy is applicable for other climacteric crops or not.

Conclusion from Objectives 2 & 3

As described in section 6.1.2.3 it was proposed that genes with independent effects from ethylene biosynthesis and also its signalling pathways via important *RIN, NOR* and *CNR* TFs could be potential candidates for combination with CrtZW and consequently improvement of ketocarotenoids level (Figure 6-2).The following crosses are suggested for future direction of improving pigments with genetic crosses strategy. For instance, *GLK* gene could be a good candidate for this means. *GLK* is the closest relative of *APRR2-Like* in tomato but distinct because of lacks the AREAEAA motif conserved in the *GLK* genes (Fitter et al., 2002). Also cv Microtom harbour the uniform mutation which, result a stop codon in the *GLK2* gene; consequently, APRR2-Like cannot affect via altering the levels of GLK2 protein (Carvalho et al., 2011). *GLK* mediated its effect by activating the expression PSI and PSII components transcripts from photosynthetic machinery (Powell et al., 2012). Interestingly neither of *GLK1* nor *GLK2* affect general ripening control transcription factors (*RIN, NOR, CNR*, and *TAGL1*) and also no effect was detected on ethylene biosynthesis and signalling genes (Nguyen et al., 2014).

*UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDB1*) also could be a good candidate for improvement of ketocarotenoids synthesis via genetic cross with CrtZW line.

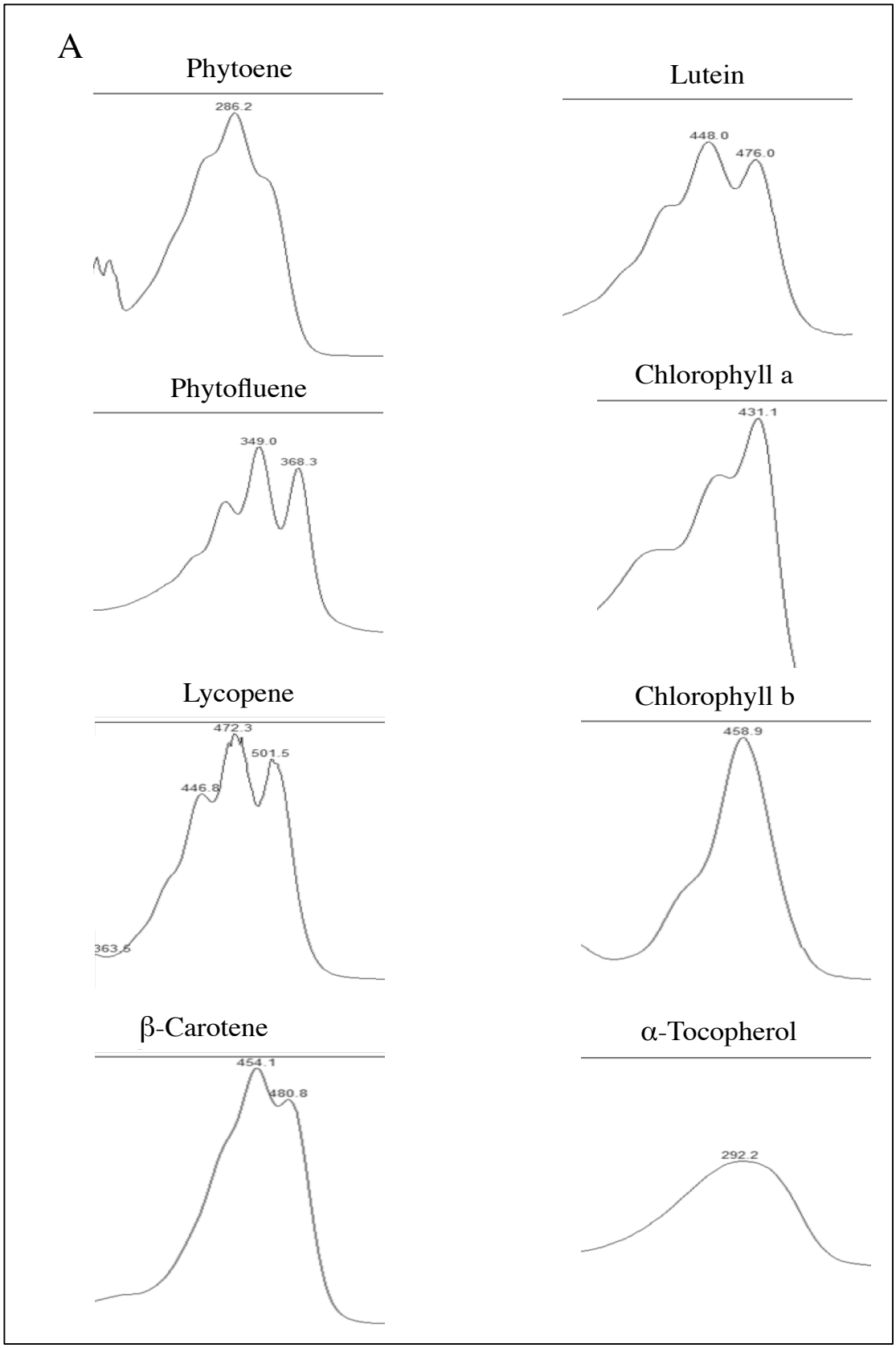
*DDB1*, which have counterpart interacts with *DET1* in Arabidopsis, is affecting via light signalling pathway (Liu et al., 2004). Its similar effect to *DET1* makes it another potential candidate for this improvement.

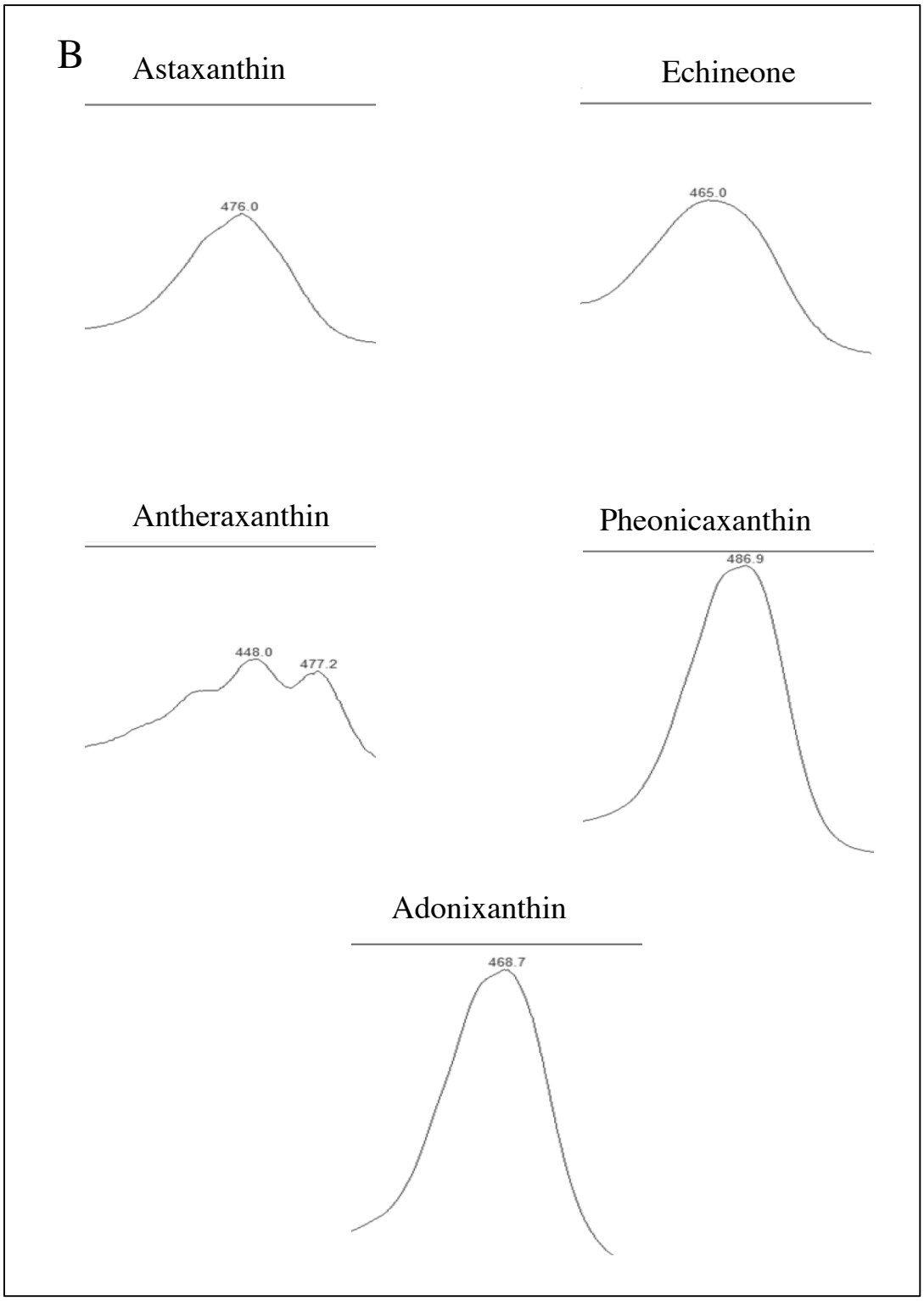
In conclusion, considering the molecular effect of each gene manipulation could provide a good insight about predicting and combining putative regulatory and TFs for enhancing the multiple metabolic and structural characteristics of crops. Moreover using more advanced methods such as system biology and CRISPR (see sections 1.7.2.3 and 1.7.2.4) could help for discovering and production of more targeted crops. System biology as explained in section 1.7.2.3 could help for discovery of potential regulatory key points and introduction of new TFs effecting regulatory pathways. Cutting edge CRISPER technique (see section 1.7.2.4) has surprising potential for example altering the germline of organisms, and modifying the genes of food crops that have traditionally been challenging for gene manipulation (Mali et al., 2013). This new method in combination with the existed wide information about effective genes could improve the gene manipulation and crop improvement much faster than before. Hope this new method could help to defend against food crises in the word.

# APPENDICES

Appendix1

Figure A 7-1 (A) Spectra of carotenoids, chlorophylls and α-tocopherol; (B) spectra of ketocarotenoids

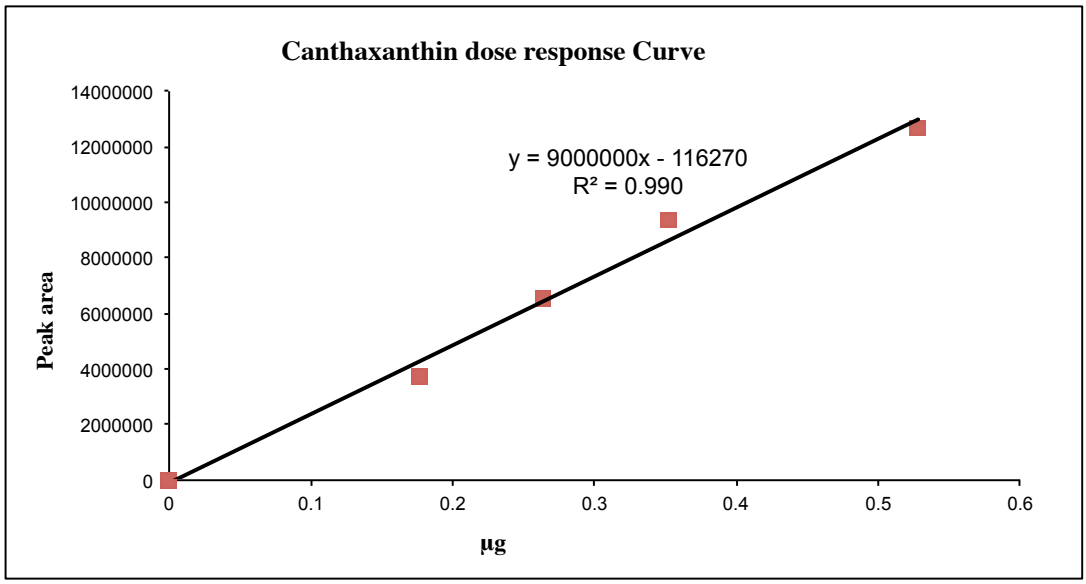



B
Astaxanthin
476.0
Echineone
465.0
Antheraxanthin
448.0
477.2
Pheonicaxanthin
486.9
Adonixanthin
468.7

Appendix2

Figure A 8-1 A) The example of canthaxanthin dose response curve and extracted equation B) Carotenoids, ketocarotenoids and α-Tocopherol dose response equations

A




B

| | |
|---|---|
| Phytoene | y=4000000x-24989 |
| Phytofluene | y=3000000x- 68968 |
| Lycopene | y=6000000x-101170 |
| Lutein | y=9000000x-462175 |
| Chlorophyll-A | y = 1958832.84x - 21131.44 |
| Chlorophyll -B | y=3000000x-13568 |
| α-Tocopherol | y=261388x-4244.7 |
| Neoxanthin | y=7000000x-182335 |
| Violaxanthin | y=8000000x-205083 |
| Astaxanthin | y=7000000x-58748 |
| Antheraxanthin | y=7000000x-202392 |
| Pheonicoxanthin | y=7000000x-58748 |
| Canthaxanthin | y=9000000x-116270 |
| Echineone | y=9000000x-195582 |
| β-Carotene | y=7000000x-408108 |

Appendix 3

Figure A 9-1 A) cDNA sequence of APRR2-Like gene with 2233 nucleotide and B) its potential homologue on chromosome 6 with 2053 nucleotide length.

**A**

Solyc08g077230.2.1 Two-component response regulator ARR11 (AHRD V1 *-*- B6UC09_MAIZE); contains Interpro domain(s) IPR006447 Myb-like DNA-binding region, SHAQKYF class

ATCTCTGTTTTCAACAGTCTCACTGTCTCCCCTAAACCCCTTACCTGACCCCCCCTTCCCTTTTA
TCTTGTTTCATTCGA
CCCACCTAACTATAATCAACAAACGACCCTTAAAAGAAGAAAAAAAAACAAGAACAGATGAGCTA
AGTCTTCTTCATTTC
CCAAGAGATACAGGATTGAATAGTTAATGACTGATTAAAAAGTGACCGAGTTGGAGGGACTAAAA
AGGATGCCTTTTTAG
AAATGATTTGCATTGAGAATGAATTATTGGGTTGGAAAGATTTCCCAAAGGGGCTTAAAGTCCTA
CTTCTTGATGAAGAT
AGCAACTCTGCTGCTGAGATGAAATCAAGGCTTGAGAAAATGGACTACATAGTCTACTCGTTCTG
CAACGAGAGCGAAGC
TTTGACTGCAATCTCAAGCAAATCCGAGGGCTTTCATGTTGCCATTGTGGAGGTAAGTGCAGGCA
ACAGTGATGGGGTTC
TACGGTTTCTTGAAAGTGCCAAAGATCTACCAACTATAATGACATCAAATATACATTCTCTTAGT
ACCATGATGAAATGT
ATTGCGCTAGGCGCAGTTGAGTTCCTTCAGAAACCATTGTCAGATGATAAACTCAAAAATATATG
GCAGCATGTAGTTCA
CAAGGCATTCAATACTAGAAAGGATGTGTCCAAATCACTTGAGCCGGTAAAAGATTCTGTCCTCT
CGATGCTGCAGTTAC
AACTAGAAATGGGTGAAGCAGATGACAAAAGTTCAAATGGAACAGAACCTCCCACTGCAGTAGCG
GAAAGCAATACTGAA
CAGTCATCGGGCTGTGATAAATACCCTGCTCCCTCAACCCCACAATTGAAACAAGGAGTGCGATC
CGTCGATGATGGTGA
CTGCCATGATCATACTATCTTCTCAACTGACCAAGACAGTGGGGAACATGATGCTGACACTAAAT
CCGTCGAAACTACTT
ATAACAATTCACTTGCTGAGAATAATGTCCAAACAAGTCCTACTGTACAGCAAGGAGATATTATT
TTGAAAGAGGATAAT
GTTTCATCTCCTGATCTAAAGACGGAGACTGATATCGCTACCACTTCACGAAGTAACGACTGCCC
TGACAATAGCATTAT
GCATTCTGCTGAACCTAGTAAAGCATCTGGTCCTCATAGTTCAAATGGGACTAAATCCAATAGGA
AGAAGATAAAGGTAG
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ATTCCTTCTCGAATA
CTGGACCTGATGAAAGTAGAGGGCTTAACGAGACATAACGTAGCTAGCCATCTCCAGAAATACAG
AATGCATCGAAAGCA
AATTTTGCCAAAGGAAGTAGAAAGAAGATGGCCTAATCCGCAACCAATAGATTCAGTCCAAAGAA
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AACCTATCATGACATTCCCACAATATCATTCTAATCATGTTGCCCCAGGTGGTCAGTTCTATCCT
GCTTGGGTAACACCA
GCAAGTTATCCGAACGGTTTACAAGTGTGGGGTTCACCTTACTATCCGGGATGGAAACCTGCAGA
GACTTGGCACTGGAC
GCCTCGTCCAGAGCTGCATGCTGATACATGGGGCTCCCCTATCATGTCACCGTCGCTTGGATCAT
ATCCACCATATCCTC
AGAATGCTGGAGTGTACCGGCCACATGGAACACATAACAGATATAGCATGCTAGAGAAGTCGTTT
GATCTTCACCCGGCG
GATGAGGTGATTGATAAAGTAGTAAAAGAGGCAATAACCAAACCATGGTTACCACTTCCTTTGGG
CCTAAAAGCTCCTTC
AACGGAGAGCGTTCTTGACGAACTTTCTAGACAAGGGATCTCAACCATTCCTTCACAAATCAACG
ACTCCCGTTGTCGGA
GATGAGATGACATGTCATTCTAATTTTTTTTGGGTCCCATAGTTGGTGCATGTCAAAAAAAAATA
ATAATCTCCAATTAC
TTGATGGACATATGTACCATGACATTACCCAGTGACCCGAGTGACCCACGCGTATGGCATTGACT
CGACGGTCAAAATCG
AGTTGTTGTAAATAATGGACCCAAATATGGGTTTTCCCTTTTTTGTTGGCCCAATTTTAGATGTT
TGGGCCGATGAGTGT
GCTCCATTAATTTCTTTGGAAAATAATGAATAAACCATTCTTAGTTTATGGGTTATAAATCCAAA
TTCTTTTT

**B**

Solyc06g061030.2.1 Two-component response regulator ARR11 (AHRD V1 *-*- B6UC09_MAIZE); contains Interpro domain(s) IPR006447 Myb-like DNA-binding region, SHAQKYF class

ATATCTCATACATATTTACCATGCATCAGAGGATGCTGAAAAGTGAGCATCATAATTAATGGCTT
GTTTGGGTGCCTGAA
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TCTTCTTGATACAGACTCCAATTTTGCCTCACAGATGAGATCAAGGCTTCAACAAATGGACTATA
TAGTTTACACATTCT
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GAGGTAAGTGCTGGC
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AACTGAGAAATATAT
GGCAGCATGTAGTTCACAAGGCATTTCATTCTGGAGGAAAGAGTGTCTCTGAGTCACTAAAGCCG
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TTGTCCATTCTAGAGCTCCAACCAGTAAAACACGAAGCAGATAATGAGAATACTAATGAAGCAGA
ACCCTTGATATCAGT
GGTGGAAAACCAAAAAGCATCATCATCCTGCTGCGATAAATATCCAGCTCCTTCAACCCCACAAC
AGAAACAAGGAGTGA
GGTCAGTGGATGATGTTGATTACCAAGATCATACTATCTTGTCAAATGAACAAGATAGTGGGATG
CATGAAGGGGACACA
AAATCTGTCGAAACTACTTCTTGCGATTCTGTTGCTGAGACTACTGTCTTGGCAGATTCTTCCGA
GCGTCTAGGAGAGGC
TATCACAAAGGAGGAACACTATTCTGCTGCTGATCAACATATGGAGGATCCTATTGCTACTTGTT
CACCAAGTAATGACA
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GCTAATAAGAAGAAA
ATGAAGGTAGACTGGACACCTGAACTACACAAGAAATTTGTTAAAGCAGTAGAGAAAATTGGTAT
AGATCAAGCCATTCC
TTCTCGAATACTGGAGCTGATGAAAGTAGAAGGCCTGACGAGACATAATATAGCTAGCCATCTCC
AGAAATTCAGGATGC
AGCGGAAGCAAATCTTGCCAAAGGAAGACGAAAGAAGGTGGCCTCGTCCTCAACCTAGAGATCCT
GTACAGAGGACCTAT
TATCCACATAAACCTGTCATGGCCTTCCCAACCCATCATTCCAACCATGCAACCACAGCTGGTCA
ATTCTATCCTGCCTG
GATACCACCAGGAGGTTACCCGAATGGTGCACATATGTGGAATTCGCCTTATTATCATGGATGGC
AACCGCCTGAGACGT
GGCACTGGAATCCTCAACCAGGGCTGTATGCTGATGTATGGGGTTGCCCCGTTACGCCTCCATCT
TTAGGATCATGTACA
CCATACCCTCAGAATGCATCTGGATTTCACAGGGCTGAGGGAATGCTGAATGGATATAGCATAAT
ACAGAAATCAGTCGA
TCTTCACCCGGCAGAGGAGGTGATAGATAAAGTGGTGAAGGAGGCAATAAACAAGCCATGGTTGC
CTCTACCCTTGGGCC
TAAAACCTCCTTCAACGGAGAGTGTTCTCGACGCGCTTTCTAAACAAGGCATCCCCGCCGTCCCT
CCACGCCACCACCGC
TCTCATCGCCCACACTGACATGCCAATTGCTTACCGGGAATCCCATATACGGAGGCTCGTGTTTG
AACACTCCACCCATG
CGCGTGGCGATGACTTCACCCGACACGAAAGAAAAAAAGTAGATCCTTTAACTGATCCGATAAAG
GTCTTGCCCGTGTGT
TGTCCCTCCTCGCTGTGCGGCACAGAATCAGCCACGTGTTTGGCTTAGATTGATCAAAAATGTTT
CGTTTTTTTAATATA
CTCAATTTATCTATAGTATTTTTTTACTAACTTTTTAAAATTACAAGTAAAAT

Appendix 4

Figure A 9-2 The cDNA alignment of APRR2-Like (Solyc08g077230.2.1) against its potential homologue on chromosome 6 (Solyc06g061030.2.1) represent three different matches which represent as A, B and C based on their length respectively.

**A**

```
 Score =  305 bits (154), Expect = 8e-82
 Identities = 594/740 (80%), Gaps = 3/740 (0%)
 Strand = Plus / Plus


Query: 1140 aaagcatctggtcctcatagttcaaatgggactaaatccaataggaagaagataaaggta 1199
            ||||| |||||||  |||||||||| |||||| ||| | |||| |||||| || ||||||
Sbjct: 990  aaagcttctggtctccatagttcaagtgggacaaaagctaataagaagaaaatgaaggta 1049


Query: 1200 gattggacacctgaactacacaagaagtttgttcaagcagtagagcaactcggtatagat 1259
            || ||||||||||||||||||||| |||||| |||||||||| || | |||||||||
Sbjct: 1050 gactggacacctgaactacacaagaaatttgttaaagcagtagagaaaattggtatagat 1109


Query: 1260 caagccattccttctcgaatactggacctgatgaaagtagagggcttaacgagacataac 1319
            |||||||||||||||||||||||||| |||||||||||| ||| | ||||||||||
Sbjct: 1110 caagccattccttctcgaatactggagctgatgaaagtagaaggcctgacgagacataat 1169


Query: 1320 gtagctagccatctccagaaatacagaatgcatcgaaagcaaattttgccaaaggaagta 1379
             |||||||||||||||||||| ||| ||||| || |||||||| ||||||||||||
Sbjct: 1170 atagctagccatctccagaaattcaggatgcagcggaagcaaatcttgccaaaggaagac 1229


Query: 1380 gaaagaagatggcctaatccgcaaccaatagattcagtccaaagaagttactatcctcat 1439
            |||||||| ||||||  ||| ||||| | |||| | || || || |  || ||||| |||
Sbjct: 1230 gaaagaaggtggcctcgtcctcaacctagagatcctgtacagaggacctattatccacat 1289


Query: 1440 aaacctatcatgacattcccacaatatcattctaatcatgttgccccaggtggtcagttc 1499
            |||||| ||||| | ||||||    ||||||| || ||||   || ||| |||||| |||
Sbjct: 1290 aaacctgtcatggccttcccaacccatcattccaaccatgcaaccacagctggtcaattc 1349


Query: 1500 tatcctgcttgggtaacaccagcaagttatccgaacggtttacaagtgtggggttcacct 1559
            |||||||| ||| || |||||| | |||| ||||| |||  |||  |||||  ||| |||
Sbjct: 1350 tatcctgcctggataccaccaggaggttacccgaatggtgcacatatgtggaattcgcct 1409


Query: 1560 tactatccgggatggaaacctgcagagacttggcactggacgcctcgtccagagctgcat 1619
            || ||||  |||||| ||||  | ||||| |||||||||  ||||  |||| |||| ||
Sbjct: 1410 tattatcatggatggcaaccgcctgagacgtggcactggaatcctcaaccagggctgtat 1469


Query: 1620 gctgatacatggggctcccctatcatgtcaccgtcgcttggatcatatccaccatatcct 1679
            ||||||  |||||| | |||  | | | | || ||  | ||||||| | ||||||| |||
Sbjct: 1470 gctgatgtatggggttgccccgttacgcctccatctttaggatcatgtacaccataccct 1529
```

```
Query: 1680 cagaatg---ctggagtgtaccggccacatggaacacataacagatatagcatgctagag 1736
            |||||||   ||||| |  || || |  | ||||  |  ||  ||||||||||  || ||
Sbjct: 1530 cagaatgcatctggatttcacagggctgagggaatgctgaatggatatagcataatacag 1589


Query: 1737 aagtcgtttgatcttcacccggcggatgaggtgattgataaagtagtaaaagaggcaata 1796
            || ||  | |||||||||||||| || |||||||| |||||||| || || |||||||||
Sbjct: 1590 aaatcagtcgatcttcacccggcagaggaggtgatagataaagtggtgaaggaggcaata 1649


Query: 1797 accaaaccatggttaccacttcctttgggcctaaaagctccttcaacggagagcgttctt 1856
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Sbjct: 1650 aacaagccatggttgcctctacccttgggcctaaaacctccttcaacggagagtgttctc 1709


Query: 1857 gacgaactttctagacaagg 1876
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Sbjct: 1710 gacgcgctttctaaacaagg 1729
```

**B**

```
 Score =  268 bits (135), Expect = 2e-70
 Identities = 354/427 (82%)
 Strand = Plus / Plus


Query: 223 aaaaggatgccttttagaaatgatttgcattgagaatgaattattgggttggaaagatt 282
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Sbjct: 79  aaaaggatgccaattcagaaatggtttgcactgagaatgaattattggagtggaaagatt 138


Query: 283 tcccaaaggggcttaaagtcctacttcttgatgaagatagcaactctgctgctgagatga 342
           ||||||| || |||||||| || |||||||||  |||   ||| | |||  |  ||||||
Sbjct: 139 tcccaaaaggacttaaagttcttcttcttgatacagactccaattttgcctcacagatga 198


Query: 343 aatcaaggcttgagaaaatggactacatagtctactcgttctgcaacgagagcgaagctt 402
            |||||||||| |  |||||||||| ||||| ||| | |||||||| ||||  || |||
Sbjct: 199 gatcaaggcttcaacaaatggactatatagtttacacattctgcaatgagaatgaggctc 258


Query: 403 tgactgcaatctcaagcaaatccgagggctttcatgttgccattgtggaggtaagtgcag 462
           || ||||||| |||||||| || ||||  || |||||||||||||| |||||||||| |
Sbjct: 259 tgtctgcaatatcaagcaagtcggaggtgttccatgttgccattgtagaggtaagtgctg 318


Query: 463 gcaacagtgatggggttctacggtttcttgaaagtgccaaagatctaccaactataatga 522
           ||||||| ||||| |   |    ||||| |||||||| ||||||||||||||||||||
Sbjct: 319 gcaacagcgatggaggattcaaatttctcgaaagtgctaaagatctaccaactataatgg 378


Query: 523 catcaaatatacattctcttagtaccatgatgaaatgtattgcgctaggcgcagttgagt 582
             ||| |||| || ||| |||  | ||||||||| ||||| || || || ||||||||||
Sbjct: 379 tttcagatattcactctattaacatcatgatgaagtgtatagcacttggtgcagttgagt 438


Query: 583 tccttcagaaaccattgtcagatgataaactcaaaaatatatggcagcatgtagttcaca 642
           |||||||||||||| |||||||||||||| | ||||||||||||||||||||||||||
Sbjct: 439 tccttcagaaaccattatcagatgataaactgagaaatatatggcagcatgtagttcaca 498


Query: 643 aggcatt 649
           |||||||
Sbjct: 499 aggcatt 505
```

**C**

```
 Score = 91.7 bits (46), Expect = 2e-17
 Identities = 124/150 (82%)
 Strand = Plus / Plus


Query: 811 gctgtgataaataccctgctccctcaaccccacaattgaaacaaggagtgcgatccgtcg 870
           |||| |||||||| || ||||| ||||||||||||  |||||||||||| | || || |
Sbjct: 670 gctgcgataaatatccagctccttcaaccccacaacagaaacaaggagtgaggtcagtgg 729


Query: 871 atgatggtgactgccatgatcatactatcttctcaactgaccaagacagtggggaacatg 930
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Sbjct: 730 atgatgttgattaccaagatcatactatcttgtcaaatgaacaagatagtgggatgcatg 789


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```

Appendix 5

Table A 9-1 Top 20 differentially expressed genes of *S.Pennellii* introgressed region between M82 and Q1968 lines at MG stage

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between M82 and Q1968 at MG stage shown as MG.M82.vs.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at MG stage presented as MG.M82.vs.Q1968.FDR Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage of development and ripening

| SOL ID | Description | GO term | MG.M82.vs.Q1968. log2FC | MG.M82.vs.Q1968. FDR |
|---|---|---|---|---|
| Solyc02g082240.1 | Unknown Protein (AHRD V1) | - | -3.621 | 2.07014E-07 |
| Solyc02g082100.2 | Neuralized (AHRD V1 *--- B4M452_DROVI); contains Interpro domain(s) IPR001841 Zinc finger, RING-type | Zinc ion binding | 3.579 | 2.07046E-82 |
| Solyc02g081890.2 | Xylosyltransferase 1 (AHRD V1 **-- B6UEF2_MAIZE); contains Interpro domain(s) IPR003406 Glycosyl transferase, family 14 | Transferase activity, transferring glycosyl groups | -3.401 | 1.14225E-06 |
| Solyc02g082120.2 | DNA-3-methyladenine glycosylase I (AHRD V1 ***-B6TPB7_MAIZE); contains Interpro domain(s) IPR005019 Methyladenine glycosylase | Base-excision repair; DNA repair | -2.940 | 3.3667E-158 |
| Solyc02g082060.1 | PPPDE peptidase domain-containing protein 1 (AHRD V1 *---PPDE1_XENLA); contains Interpro domain(s) IPR008580 Protein of unknown function DUF862, eukaryotic | - | -2.079 | 4.57859E-70 |
| Solyc02g082190.2 | CTP synthase (AHRD V1 **** B6U7R1_MAIZE); contains Interpro domain(s) IPR004468 CTP synthase | CTP synthase activity; identical protein binding | -2.041 | 7.51455E-97 |
| Solyc02g081930.2 | Unknown Protein (AHRD V1) | - | -1.826 | 5.24026E-18 |
| Solyc02g082210.2 | Genomic DNA chromosome 3 P1 clone MOJ10 (AHRD V1 ***- | - | 1.425 | 3.88769E-34 |

| | | | | |
|---|---|---|---|---|
| | Q9LSE0_ARATH) | | | |
| Solyc02g082180.2 | DNA replication licensing factor (AHRD V1 ***-Q2TWS7_ASPOR); contains Interpro domain(s) IPR008049 MCM protein 6 | Identical protein binding | -1.386 | 2.68751E-35 |
| Solyc02g081860.1 | Pentatricopeptide repeat-containing protein (AHRD V1 ***-D7MRA3_ARALY); contains Interpro domain(s) IPR002885 Pentatricopeptide repeat | - | -1.359 | 4.44582E-18 |
| Solyc02g082050.2 | Tir-nbs-lrr, resistance protein | LRR domain binding; nucleotide binding | -1.293 | 0.003820904 |
| Solyc02g081850.2 | Amino acid transporter (AHRD V1 **** C2SWS0_BACCE); contains Interpro domain(s) IPR015606 Cationic amino acid transporter | High affinity arginine transmembrane transporter activity; cationic amino acid transmembrane transporter activity | -1.233 | 1.04169E-23 |
| Solyc02g081880.2 | Molybdenum cofactor sulfurase protein-Like (AHRD V1 **--Q653D7_ORYSJ); contains Interpro domain(s) IPR005303 MOSC, N-terminal beta barrel | Phospholipid-translocating ATPase activity | 1.230 | 4.11849E-44 |
| Solyc02g081830.2 | HAD-superfamily hydrolase (AHRD V1 ***- D5A3P4_SPIPL); contains Interpro domain(s) IPR011949 HAD-superfamily hydrolase, subfamily IA, REG-2-Like | Protein binding | -1.176 | 7.30105E-17 |
| Solyc02g082150.1 | Unknown Protein (AHRD V1) | - | 1.162 | 0.036783099 |
| Solyc02g082140.2 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR008166 Protein of unknown function DUF23 | - | 1.155 | 2.64789E-34 |
| Solyc02g082070.2 | Cytochrome P450 | Oxygen binding | -1.123 | 2.42264E-24 |
| Solyc02g081970.2 | Vacuolar sorting receptor 7 (AHRD V1 **** B6UEQ5_MAIZE); contains Interpro domain(s) IPR003137 Protease-associated PA | Protein binding; mino-terminal vacuolar sorting propeptide binding | 1.090 | 1.95994E-28 |

| | | | | |
|---|---|---|---|---|
| Solyc02g082110.2 | Pre-mRNA-splicing factor cwc24 (AHRD V1 **--B2WLF6_PYRTR); contains Interpro domain(s) IPR000571 Zinc finger, CCCH-type | Zinc ion binding | -1.067 | 1.05382E-41 |
| Solyc02g082160.1 | Pentatricopeptide repeat-containing protein (AHRD V1 ***-D7KMD0_ARALY); contains Interpro domain(s) IPR002885 Pentatricopeptide repeat | Endonuclease activity | -0.989 | 3.5873E-08 |

Appendix 6

Table A 9-2 Top 20 differentially expressed genes of *S.Pennellii* introgressed region between M82 and Q1968 lines at 3dpb stage

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between M82 and Q1968 at 3 dpb shown as BR3.M82.vs.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at 3dpb presented as BR3.M82.vs.Q1968.FDR. Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage of development and ripening

| GENE ID | Description | GO term | BR3.M82.vs.Q1968 .log2FC | BR3.M82.vs.Q1968 .FDR |
|---|---|---|---|---|
| Solyc02g082240.1 | Unknown Protein (AHRD V1) | - | 3.120 | 0.034 |
| Solyc02g081800.1 | Acyltransferase (Fragment) (AHRD V1 **-- C1JZ77_9SOLA); contains Interpro domain(s) IPR003480 Transferase | Transferase activity | 1.561 | 0.001 |
| Solyc02g082260.2 | Hydroxy-methylglutaryl-coenzyme A reductase (AHRD V1 **** O48624_TOBAC); contains Interpro domain(s) IPR004554 Hydroxymethylglutaryl-CoA reductase, class I, catalytic | Hydroxymethylglutaryl-CoA reductase activity | -0.981 | 0.000 |
| Solyc02g081680.2 | Nucleolar complex protein 2 homolog (AHRD V1 ***- C0H9R3_SALSA); contains Interpro domain(s) IPR005343 Uncharacterised protein family UPF0120 | Identical protein binding | -0.715 | 0.000 |
| Solyc02g081830.2 | HAD-superfamily hydrolase (AHRD V1 ***- D5A3P4_SPIPL); contains Interpro domain(s) IPR011949 HAD-superfamily hydrolase, subfamily IA, REG-2-Like | Protein binding | -0.701 | 0.000 |
| Solyc02g081980.2 | Ectonucleoside triphosphate diphosphohydrolase 6 (AHRD V1 **-- Q6YHK4_CAVPO); contains Interpro domain(s) IPR000407 Nucleoside phosphatase GDA1/CD39 | ATPase activity; guanosine-diphosphatase activity | -0.645 | 0.046 |

| | | | | |
|---|---|---|---|---|
| Solyc02g082180.2 | DNA replication licensing factor (AHRD V1 ***-Q2TWS7_ASPOR); contains Interpro domain(s) IPR008049 MCM protein 6 | Identical protein binding | -0.626 | 0.000 |
| Solyc02g081970.2 | Vacuolar sorting receptor 7 (AHRD V1 **** B6UEQ5_MAIZE); contains Interpro domain(s) IPR003137 Protease-associated PA | Protein binding; mino-terminal vacuolar sorting propeptide binding | 0.613 | 0.000 |
| Solyc02g082290.2 | MORC family CW-type zinc finger 3 (AHRD V1 **--Q4VBZ9_HUMAN); contains Interpro domain(s) IPR003594 ATP-binding region, ATPase-Like | ATP binding; zinc ion binding | -0.612 | 0.001 |
| Solyc02g082120.2 | DNA-3-methyladenine glycosylase I (AHRD V1 ***-B6TPB7_MAIZE); contains Interpro domain(s) IPR005019 Methyladenine glycosylase | Base-excision repair; DNA repair | -0.596 | 0.036 |
| Solyc02g082060.1 | PPPDE peptidase domain-containing protein 1 (AHRD V1 *---PPDE1_XENLA); contains Interpro domain(s) IPR008580 Protein of unknown function DUF862, eukaryotic | - | 0.449 | 0.008 |
| Solyc02g082190.2 | CTP synthase (AHRD V1 **** B6U7R1_MAIZE); contains Interpro domain(s) IPR004468 CTP synthase | CTP synthase activity; identical protein binding | -0.436 | 0.000 |
| Solyc02g081810.2 | tRNA pseudouridine synthase B (AHRD V1 *-*- TRUB_PELTS); contains Interpro domain(s) IPR004802 Pseudouridine synthase, putative | Protein domain specific binding | -0.432 | 0.000 |
| Solyc02g082280.2 | Genomic DNA chromosome 5 TAC clone K2A18 (AHRD V1 **--Q9FKY7_ARATH); contains Interpro domain(s) IPR012880 Protein of unknown function DUF1683, C-terminal | - | -0.415 | 0.000 |
| Solyc02g081850.2 | Amino acid transporter (AHRD V1 **** C2SWS0_BACCE); | High affinity arginine | -0.387 | 0.027 |

| | | | | |
|---|---|---|---|---|
| | contains Interpro domain(s) IPR015606 Cationic amino acid transporter | transmembrane transporter activity; cationic amino acid transmembrane transporter activity | | |
| Solyc02g081660.2 | GTP-binding protein era homolog (AHRD V1 ***-D7AAD9_STAND); contains Interpro domain(s) IPR005662 GTP-binding protein Er | GTPase activity | -0.368 | 0.007 |
| Solyc02g081940.2 | RNA binding protein (AHRD V1 **** D8LH81_ECTSI); contains Interpro domain(s) IPR012677 Nucleotide-binding, alpha-beta plait | Helicase activity; mRNA binding | -0.345 | 0.002 |
| Solyc02g081700.1 | Proteasome subunit alpha type (AHRD V1 ***- Q38HT0_SOLTU); contains Interpro domain(s) IPR001353 Proteasome, subunit alpha/beta | Proteasome core complex, alpha-subunit complex; proteasome core complex | 0.304 | 0.004 |
| Solyc02g081880.2 | Molybdenum cofactor sulfurase protein-like (AHRD V1 **--Q653D7_ORYSJ); contains Interpro domain(s) IPR005303 MOSC, N-terminal beta barrel | phospholipid-translocating ATPase activity | -0.267 | 0.007 |
| Solyc02g082130.1 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR007019 Surfeit locus 6 | - | -0.262 | 0.009 |

Appendix 7

Table A 9-3 Top 20 differentially expressed genes of *S.Pennellii* introgressed region between M82 and Q1968 lines at 7dpb stage

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between M82 and Q1968 at 7 dpb shown as BR7.M82.vs.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at 7 dpb presented as BR7.M82.vs.Q1968.FDR Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage.

| SOL ID | Description | GO term | BR7.M82.vs.Q1968.log2FC | BR7.M82.vs.Q1968.FDR |
|---|---|---|---|---|
| Solyc02g082100.2 | Neuralized (AHRD V1 *--- B4M452_DROVI); contains Interpro domain(s) IPR001841 Zinc finger, RING-type | Zinc ion binding | 0.735 | 0.000 |
| Solyc02g082120.2 | DNA-3-methyladenine glycosylase I (AHRD V1 ***-B6TPB7_MAIZE); contains Interpro domain(s) IPR005019 Methyladenine glycosylase | Base-excision repair; DNA repair | 0.728 | 0.002 |
| Solyc02g082260.2 | Hydroxy-methylglutaryl-coenzyme A reductase (AHRD V1 **** O48624_TOBAC); contains Interpro domain(s) IPR004554 Hydroxymethylglutaryl-CoA reductase, class I, catalytic | Hydroxymethylglutaryl-CoA reductase activity | 0.707 | 0.000 |
| Solyc02g082070.2 | Cytochrome P450 | Oxygen binding | 0.520 | 0.005 |
| Solyc02g081860.1 | Pentatricopeptide repeat-containing protein (AHRD V1 ***-D7MRA3_ARALY); contains Interpro domain(s) IPR002885 Pentatricopeptide repeat | - | -0.504 | 0.004 |
| Solyc02g082290.2 | MORC family CW-type zinc finger 3 (AHRD V1 **--Q4VBZ9_HUMAN); contains Interpro domain(s) IPR003594 ATP-binding region, ATPase-Like | ATP binding; zinc ion binding | -0.492 | 0.005 |

| | | | | |
|---|---|---|---|---|
| Solyc02g082140.2 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR008166 Protein of unknown function DUF23 | - | 0.478 | 0.000 |
| Solyc02g081830.2 | HAD-superfamily hydrolase (AHRD V1 ***- D5A3P4_SPIPL); contains Interpro domain(s) IPR011949 HAD-superfamily hydrolase, subfamily IA, REG-2-Like | Protein binding | -0.416 | 0.004 |
| Solyc02g081850.2 | Amino acid transporter (AHRD V1 **** C2SWS0_BACCE); contains Interpro domain(s) IPR015606 Cationic amino acid transporter | High affinity arginine transmembrane transporter activity; cationic amino acid transmembrane transporter activity | -0.400 | 0.012 |
| Solyc02g081700.1 | Proteasome subunit alpha type (AHRD V1 ***- Q38HT0_SOLTU); contains Interpro domain(s) IPR001353 Proteasome, subunit alpha/beta | Proteasome core complex, alpha-subunit complex; proteasome core complex | -0.394 | 0.000 |
| Solyc02g082190.2 | CTP synthase (AHRD V1 **** B6U7R1_MAIZE); contains Interpro domain(s) IPR004468 CTP synthase | CTP synthase activity; identical protein binding | -0.384 | 0.002 |
| Solyc02g082130.1 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR007019 Surfeit locus 6 | - | -0.353 | 0.000 |
| Solyc02g082110.2 | Pre-mRNA-splicing factor cwc24 (AHRD V1 **-- B2WLF6_PYRTR); contains Interpro domain(s) IPR000571 Zinc finger, CCCH-type | Zinc ion binding | -0.325 | 0.007 |
| Solyc02g082200.2 | Glutaredoxin (AHRD V1 ***- A9LC71_PTEVI); contains Interpro domain(s) IPR004480 Glutaredoxin-related protein | Cell redox homeostasis | -0.308 | 0.002 |
| Solyc02g082270.2 | RNA-binding protein 68390-68829 (AHRD V1 *--* Q9C805_ARATH); contains Interpro domain(s) IPR000504 RNA recognition motif, RNP-1 | Poly (U) RNA binding | -0.304 | 0.001 |
| Solyc02g081680.2 | Nucleolar complex protein 2 homolog (AHRD V1 ***-C0H9R3_SALSA); contains Interpro domain(s) IPR005343 Uncharacterised protein family UPF0120 | Identical protein binding | -0.292 | 0.002 |
| Solyc02g081840.2 | MMP37-Like protein mitochondrial (AHRD V1 ***- C1C198_9MAXI); | Protein binding | -0.274 | 0.034 |

| | | | | |
|---|---|---|---|---|
| | contains Interpro domain(s) IPR015222 Mitochondrial matrix Mmp37 | | | |
| Solyc02g081960.2 | Splicing factor (AHRD V1 *-*- Q5CJR5_CRYHO); contains Interpro domain(s) IPR000061 SWAP/Surp | Protein binding; RNA binding | -0.269 | 0.026 |
| Solyc02g081880.2 | Molybdenum cofactor sulfurase protein-like (AHRD V1 **-- Q653D7_ORYSJ); contains Interpro domain(s) IPR005303 MOSC, N-terminal beta barrel | Phospholipid-translocating ATPase activity | 0.265 | 0.006 |
| Solyc02g082220.2 | Protein transport protein SEC24 (AHRD V1 ***- C5FL43_NANOT); contains Interpro domain(s) IPR006896 Sec23/Sec24 trunk region | Protein binding | -0.239 | 0.031 |

Appendix 8

Table A 9-4 Top differentially expressed genes between M82 and Q1968 at MG stage in the overall transcriptome

The Gene ID represents the gene names from Sol Genomics Network. GO term indicates the Gene Ontology. Multiple testing corrections was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between M82 and Q1968 at MG is shown as MG.M82.vs.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at MG stage presented as MG.M82.vs.Q1968.FDR Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage af development and ripening.

| Gene | Description | GO terms | MG.M82.vs.Q1968.log2FC | MG.M82.vs.Q1968.FDR |
|---|---|---|---|---|
| Solyc03g119590.1 | NIMIN2c protein (AHRD V1 ***- A0FJY4_TOBAC) | - | 11.265 | 2E-141 |
| Solyc01g106980.1 | Endo-1 4-beta-xylanase (AHRD V1 *--- B6SW51_MAIZE); contains Interpro domain(s) IPR013781 Glycoside hydrolase, subgroup, catalytic core | Protein binding | -10.859 | 5E-54 |
| Solyc04g025240.2 | GCN5-related N-acetyltransferase (GNAT) family protein-like (AHRD V1 ***-Q6K836_ORYSJ) | - | -9.878 | 2E-56 |
| Solyc07g065570.1 | Nuclear transcription factor Y subunit B-6 (AHRD V1 *-*- B6UH02_MAIZE); contains Interpro domain(s) IPR003957 Transcription factor, CBFA/NFYB, DNA topoisomerase | DNA binding<br>intracellular | -9.815 | 1E-80 |
| Solyc02g091180.1 | cDNA clone J100026I16 full insert sequence (AHRD V1 **-- B7FA06_ORYSJ) | - | 9.797 | 2E-105 |
| Solyc08g014430.2 | Formin 3 (AHRD V1 *-*- D0QAN4_ARATH); contains Interpro domain(s) IPR015425 Actin-binding FH2 | Actin cytoskeleton organization | -9.419 | 4E-180 |
| Solyc07g052790.1 | Nbs-lrr, resistance protein | LRR domain binding<br>nucleotide binding | 9.347 | 1E-49 |
| Solyc01g105920.2 | (E)-beta-ocimene synthase (AHRD V1 **** Q5CD81_CITUN); contains Interpro domain(s) IPR005630 Terpene synthase, metal-binding domain | (E)-beta-ocimene synthase activity<br>myrcene synthase activity | -8.900 | 6E-18 |
| Solyc02g084370.1 | Receptor like kinase, RLK | Transmembrane receptor protein serine/threonine kinase activity | 8.611 | 4E-21 |

| | | | | |
|---|---|---|---|---|
| Solyc02g079700.1 | S-receptor kinase (AHRD V1 ***- Q43393_BRANA) | - | 8.586 | 4E-18 |
| Solyc07g043500.1 | UDP-glucosyltransferase (AHRD V1 ***- Q8LKG3_STERE); contains Interpro domain(s) IPR002213 UDP-glucuronosyl/UDP-glucosyltransferase | Metabolic process | 8.523 | 1E-111 |
| Solyc03g095940.1 | LOB domain family protein (AHRD V1 ***- D7L9U4_ARALY); contains Interpro domain(s) IPR004883 Lateral organ boundaries, LOB | Protein binding | 8.469 | 6E-30 |
| Solyc01g068410.2 | Auxin Efflux Carrier (AHRD V1 **** Q673E6_MEDTR); contains Interpro domain(s) IPR004776 Auxin efflux carrier | Auxin: hydrogen symporter activity | 8.399 | 8E-07 |
| Solyc03g097580.2 | MtN3-Like protein (AHRD V1 **-- Q9LUE3_ARATH); contains Interpro domain(s) IPR018179 RAG1-activating protein 1 homologue | Integral to membrane | 8.389 | 3E-17 |
| Solyc01g014560.2 | D-mannose binding lectin family protein expressed (AHRD V1 **-- Q2RAY8_ORYSJ); contains Interpro domain(s) IPR001480 Curculin-like (mannose-binding) lectin | Sugar binding (obsolete GO:sugar binding) | -8.100 | 5E-112 |
| Solyc03g093120.2 | Xyloglucan endotransglucosylase/hydrolase 9 (AHRD V1 **** C0IRG8_9ERIC); contains Interpro domain(s) IPR016455 Xyloglucan endotransglucosylase/hydrolase | Hydrolase activity, acting on glycosyl bonds /xyloglucan: xyloglucosyl transferase activity | 8.099 | 2E-96 |
| Solyc02g085800.1 | Acetyl esterase (AHRD V1 **-- AES_SHIF8); contains Interpro domain(s) IPR013094 Alpha/beta hydrolase fold-3 | Lipase activity<br>serine hydrolase activity | -8.082 | 4E-59 |
| Solyc01g087590.2 | Polyamine oxidase (AHRD V1 **** Q4H439_TOBAC); contains Interpro domain(s) IPR002937 Amine oxidase | Polyamine oxidase activity<br>flavin adenine dinucleotide binding | 8.066 | 9E-58 |
| Solyc01g007030.2 | U-box domain-containing protein (AHRD V1 ***- D7LU64_ARALY); contains Interpro domain(s) IPR016024 Armadillo-type fold | - | 8.009 | 6E-19 |
| Solyc02g080220.2 | Pectinesterase (AHRD V1 ***- B9RXQ4_RICCO); contains Interpro domain(s) IPR000070 Pectinesterase, catalytic | Cell wall<br>pectinesterase activity | -7.995 | 0E+00 |
| Solyc02g061990.2 | Basic leucine zipper transcription factor (AHRD V1 **-* Q9FQ01_9ROSI) | Protein binding<br>transcription activator activity | 7.928 | 2E-20 |

| | | | | |
|---|---|---|---|---|
| | | (obsolete GO:0016563) | | |
| Solyc03g093130.2 | Xyloglucan endotransglucosylase/hydrolase 9 (AHRD V1 **** C0IRG8_9ERIC); contains Interpro domain(s) IPR016455 Xyloglucan endotransglucosylase/hydrolase IPR008263 Glycoside hydrolase, family 16, active site | Hydrolase activity, acting on glycosyl bonds/ xyloglucan: xyloglucosyl transferase activity | 7.920 | 6E-110 |
| Solyc05g055220.1 | Plant-specific domain TIGR01568 family protein (AHRD V1 **-- B6U9N9_MAIZE); contains Interpro domain(s) IPR006458 Protein of unknown function DUF623, plant | - | 7.913 | 9E-18 |

Appendix 9

Table A 9-5 Top differentially expressed genes between M82 and Q1968 at 3dpb stage in the overall transcriptome

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between M82 and Q1968 at 3 dpb shown as BR3.M82.vs.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at 3dpb presented as BR3.M82.vs.Q1968.FDR. Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage.

| Gene | Description | GO terms | BR3.M82.vs.Q1968.log2FC | BR3.M82.vs.Q1968.FDR |
|---|---|---|---|---|
| Solyc02g062770.1 | Late embryogenesis abundant protein (AHRD V1 *-*- Q3I3Y9_PICGL) | - | -11.649 | 2E-12 |
| Solyc01g009660.1 | Low-temperature-induced 65 kDa protein (AHRD V1 *-*- LTI65_ARATH) | - | -10.913 | 1E-11 |
| Solyc01g081590.2 | Non-specific lipid-transfer protein (AHRD V1 **** B9N6K5_POPTR); contains Interpro domain(s) IPR013770 Plant lipid transfer protein and hydrophobic protein, helical | Calmodulin binding lipid transporter activity | -10.774 | 8E-17 |
| Solyc01g098770.1 | Xylanase inhibitor (Fragment) (AHRD V1 **-- Q53IQ4_WHEAT); contains Interpro domain(s) IPR001461 Peptidase A1 | Proteolysis | -9.699 | 6E-11 |
| Solyc01g090360.2 | Non-specific lipid-transfer protein (AHRD V1 ***- B9RD07_RICCO); contains Interpro domain(s) IPR013770 Plant lipid transfer protein and hydrophobic protein, helical | Lipid transport | -9.123 | 7E-11 |
| Solyc04g064710.2 | Alcohol dehydrogenase 2 (AHRD V1 **** Q9FZ01_VITVI); contains Interpro domain(s) IPR002085 Alcohol dehydrogenase superfamily, zinc-containing | Alcohol dehydrogenase activity, zinc-dependent | -8.102 | 2E-08 |
| Solyc02g090370.2 | Reticulon-like protein B13 (AHRD V1 ***- RTNLM_ARATH); contains Interpro domain(s) IPR003388 Reticulon | Endoplasmic reticulum | -7.972 | 1E-07 |
| Solyc01g007940.2 | Alanine aminotransferase 2 (AHRD V1 **** A8IKE5_SOYBN); contains Interpro domain(s) IPR004839 Aminotransferase, class I and II | L-alanine:2-oxoglutarate aminotransferase activity | -7.788 | 1E-04 |
| Solyc02g077530.1 | O-methyltransferase (AHRD V1 ***- A5HK00_VITVI); contains Interpro domain(s) IPR016461 O-methyltransferase, COMT, eukaryota | O-methyltransferase activity protein dimerization activity | -7.744 | 2E-06 |

| | | | | |
|---|---|---|---|---|
| Solyc02g084840.2 | Dehydrin DHN1 (AHRD V1 *-*- DHN1_PEA); contains Interpro domain(s) IPR000167 Dehydrin | Response to water stimulus | -7.694 | 1E-28 |
| Solyc02g079560.1 | Receptor-like protein kinase (AHRD V1 ***- Q9ZT06_ARATH); contains Interpro domain(s) IPR001245 Tyrosine protein kinase | Protein phosphorylation | -7.651 | 6E-12 |
| Solyc01g009470.1 | Poly (AHRD V1 ***- B9SCR8_RICCO); contains Interpro domain(s) IPR012317 Poly(ADP-ribose) polymerase, catalytic regio | Nucleus protein ADP-ribosylation/ NAD+ ADP-ribosyltransferase activity/ intracellular | -7.623 | 2E-08 |
| Solyc09g082340.2 | Vicilin-like protein (Fragment) (AHRD V1 **-- Q9SEW4_9ROSI); contains Interpro domain(s) IPR014710 RmlC-Like jelly roll fold | Nutrient reservoir activity | -7.581 | 5E-25 |
| Solyc09g072560.2 | Legumin 11S-globulin (AHRD V1 **-- Q39770_GINBI); contains Interpro domain(s) IPR014710 RmlC-Like jelly roll fold | Nutrient reservoir activity | -7.530 | 2E-14 |
| Solyc06g034040.1 | Oleosin (AHRD V1 ***- A5JVA7_FICAW); contains Interpro domain(s) IPR000136 Oleosin | Integral to membrane | -7.416 | 3E-10 |
| Solyc03g096040.2 | Mitochondrial peroxiredoxin (1-Cys Prx) with thioredoxin peroxidase activity (AHRD V1 **** C4QX37_PICPG); contains Interpro domain(s) IPR000866 Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen | Thioredoxin peroxidase activity glutathione peroxidase activity | -7.393 | 6E-15 |
| Solyc01g005230.2 | S-adenosyl-L-methionine salicylic acid carboxyl methyltransferase (AHRD V1 **** A7XZE9_9MAGN); contains Interpro domain(s) IPR005299 SAM dependent carboxyl methyltransferase | S-adenosylmethionine-dependent methyltransferase activity | -7.360 | 4E-07 |
| Solyc05g017790.1 | Unknown Protein (AHRD V1) | - | 7.331 | 1E-09 |
| Solyc09g025210.2 | Legumin 11S-globulin (AHRD V1 **-- Q39770_GINBI); contains Interpro domain(s) IPR014710 RmlC-Like jelly roll fold | Nutrient reservoir activity | -7.329 | 7E-22 |
| Solyc02g077980.1 | Unknown Protein (AHRD V1) | - | -7.270 | 1E-06 |
| Solyc09g025210.2 | Legumin 11S-globulin (AHRD V1 **-- Q39770_GINBI); contains Interpro domain(s) IPR014710 RmlC-Like jelly roll fold | Nutrient reservoir activity | -7.329 | 7E-22 |
| Solyc01g017840.1 | subunit of replication protein A (AHRD V1 *-*- A6BLP0_IPONI); contains Interpro domain(s) IPR016027 Nucleic acid-binding, OB-fold-Like | - | -7.238 | 1E-74 |

Appendix 10

Table A 9-6 Top differentially expressed genes between M82 and Q1968 at 7dpb stage in the overall transcriptome

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The log2 fold change (log2FC) between M82 and Q1968 at 7 dpb shown as BR7.M82.vs.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction at 7 dpb presented as BR7.M82.vs.Q1968.FDR Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage.

| Gene | Description | GO terms | BR7.M82.vs.Q1968 .log2FC | BR7.M82.vs.Q1968 .FDR |
|---|---|---|---|---|
| Solyc02g062770.1 | Late embryogenesis abundant protein (AHRD V1 *-*- Q3I3Y9_PICGL) | - | 11.203 | 2E-14 |
| Solyc01g065860.1 | Helitron helicase-like protein (AHRD V1 ***- D0NDZ2_PHYIN); contains Interpro domain(s) IPR010285 Protein of unknown function DUF889, eukaryote | - | -9.938 | 5E-54 |
| Solyc02g084850.2 | Unknown Protein (AHRD V1); contains Interpro domain(s) IPR000167 Dehydrin | Response to water stimulus | 9.166 | 2E-40 |
| Solyc02g081600.2 | Zinc transporter protein (AHRD V1 **** D5LMF9_9FABA); contains Interpro domain(s) IPR004698 Zinc/iron permease, fungal and plant | low-affinity zinc ion transmembrane transporter activity | 8.949 | 7E-12 |
| Solyc02g079710.2 | Serine/threonine kinase receptor (AHRD V1 **** Q7DMS5_BRANA); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase | Transmembrane receptor protein kinase activity protein serine/threonine kinase activity | 8.574 | 2E-23 |
| Solyc03g005550.2 | Serine/threonine-protein phosphatase 7 long form homolog (AHRD V1 **-- PPP7L_ARATH); contains Interpro domain(s) IPR019557 Aminotransferase-like, plant mobile domain | - | 8.469 | 3E-03 |
| Solyc02g090370.2 | Reticulon-like protein B13 (AHRD V1 ***- RTNLM_ARATH); contains Interpro domain(s) IPR003388 Reticulon | Endoplasmic reticulum | 8.117 | 1E-07 |
| Solyc04g025240.2 | GCN5-related N-acetyltransferase (GNAT) family protein-like (AHRD V1 ***- | - | -8.028 | 4E-13 |

| | | | | |
|---|---|---|---|---|
| | Q6K836_ORYSJ) | | | |
| Solyc02g079560.1 | Receptor-like protein kinase (AHRD V1 ***- Q9ZT06_ARATH); contains Interpro domain(s) IPR001245 Tyrosine protein kinase | Protein phosphorylation | -7.627 | 6E-14 |
| Solyc02g079800.1 | Unknown Protein (AHRD V1) | - | -7.389 | 4E-09 |
| Solyc04g072470.2 | Defensin-like protein (AHRD V1 ***- Q948T4_PYRPY); contains Interpro domain(s) IPR008176 Gamma thionin | Peptidase inhibitor activity | 7.342 | 5E-07 |
| Solyc01g007940.2 | Alanine aminotransferase 2 (AHRD V1 **** A8IKE5_SOYBN); contains Interpro domain(s) IPR004839 Aminotransferase, class I and II | L-alanine:2-oxoglutarate aminotransferase activity | 7.301 | 7E-05 |
| Solyc03g083230.1 | Helicase-like protein (AHRD V1 *-*- Q9AYF0_ORYSJ); contains Interpro domain(s) IPR010285 Protein of unknown function DUF889, eukaryote | - | -7.228 | 2E-76 |
| Solyc02g079550.1 | Serine/threonine kinase receptor (AHRD V1 **** Q7DMS5_BRANA); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase | Transmembrane receptor protein kinase activity protein serine/threonine kinase activity | -7.218 | 4E-45 |
| Solyc00g067470.1 | Unknown Protein (AHRD V1) | | -7.105 | 1E-07 |
| Solyc01g005150.2 | Cytochrome b561 (AHRD V1 ***- Q3LGX5_CITLA); contains Interpro domain(s) IPR004877 Cytochrome b561, eukaryote | Ferric-chelate reductase activity | -7.105 | 1E-03 |
| Solyc01g090360.2 | Non-specific lipid-transfer protein (AHRD V1 ***- B9RD07_RICCO); contains Interpro domain(s) IPR013770 Plant lipid transfer protein and hydrophobic protein, helical | Lipid transport | 7.088 | 5E-13 |
| Solyc01g011430.2 | Diacylglycerol O-acyltransferase (AHRD V1 ***- A5UWY0_ROSS1); contains Interpro domain(s) IPR009721 Protein of unknown function DUF1298 | - | 6.996 | 2E-05 |
| Solyc07g032800.1 | Replication protein A DNA-binding subunit (AHRD V1 ***- B6SL03_MAIZE); contains Interpro domain(s) IPR012340 Nucleic acid-binding, OB-fold | - | -6.951 | 3E-56 |
| Solyc01g080030.1 | Oxidoreductase zinc-binding dehydrogenase family (AHRD V1 **-- B4WZD2_9GAMM); contains Interpro domain(s) IPR002085 Alcohol dehydrogenase superfamily, zinc-containing | Metabolic process oxidation-reduction process | 6.794 | 7E-09 |

| | | | | |
|---|---|---|---|---|
| Solyc01g017840.1 | subunit of replication protein A (AHRD V1 *-*- A6BLP0_IPONI); contains Interpro domain(s) IPR016027 Nucleic acid-binding, OB-fold-like | - | -6.780 | 4E-138 |

Appendix 11

Table A 9-7 Top differentially expressed genes between M82 and Q1968 in all of stages in the overall transcriptome

The Gene ID represents the gene names from Sol Genomics Network. GO term indicate the Gene Ontology. Multiple testing correction was performed using the Benjamini-Hochberg (BH) method. The overall log2 fold change (log2FC) between M82 and Q1968 shown as All.M82.vs.All.Q1968.log2FC and the false discovery rate (FDR) calculated with the Benjamini-Hochberg correction presented as All.M82.vs.All.Q1968.FDR Genes were considered statistically significantly differentially expressed if the false discovery rate was less than 0.05. The RNAseq reads performed from three RNA extractions from a pool of minimum 9 fruits of three biological replicates at each stage. In this analysis all of the data for three stages of MG, 3dpb and 7dpb of M82 considered against the same data of Q1968.

| Gene | Description | GO terms | All.M82.vs.All.Q1968.log2FC | All.M82.vs.All.Q1968.FDR |
|---|---|---|---|---|
| Solyc04g025240.2 | GCN5-related N-acetyltransferase (GNAT) family protein-like (AHRD V1 ***-Q6K836_ORYSJ) | | -24.040 | 2E-57 |
| Solyc02g079560.1 | Receptor-like protein kinase (AHRD V1 ***- Q9ZT06_ARATH); contains Interpro domain(s) IPR001245 Tyrosine protein kinase | Protein phosphorylation | -19.119 | 1E-23 |
| Solyc01g017850.1 | Helicase-like protein (AHRD V1 *-*- Q9AYF0_ORYSJ); contains Interpro domain(s) IPR010285 Protein of unknown function DUF889, eukaryot | | -18.571 | 2E-75 |
| Solyc01g017840.1 | Subunit of replication protein A (AHRD V1 *-*- A6BLP0_IPONI); contains Interpro domain(s) IPR016027 Nucleic acid-binding, OB-fold-Like | | -15.952 | 3E-79 |
| Solyc00g067470.1 | Unknown Protein (AHRD V1) | | -18.086 | 3E-36 |
| Solyc02g079560.1 | Receptor-like protein kinase (AHRD V1 ***- Q9ZT06_ARATH); contains Interpro domain(s) IPR001245 Tyrosine protein kinase | Protein phosphorylation | -19.119 | 1E-23 |
| Solyc03g083230.1 | Helicase-like protein (AHRD V1 *-*- Q9AYF0_ORYSJ); contains Interpro domain(s) IPR010285 Protein of unknown function DUF889, eukaryote | | -17.417 | 2E-39 |
| Solyc02g079550.1 | Serine/threonine kinase receptor (AHRD V1 **** Q7DMS5_BRANA); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase | Transmembrane receptor protein kinase activity protein serine/threonine | -16.988 | 5E-75 |

| | | | | |
|---|---|---|---|---|
| | | kinase activity | | |
| Solyc02g081600.2 | Zinc transporter protein (AHRD V1 **** D5LMF9_9FABA); contains Interpro domain(s) IPR004698 Zinc/iron permease, fungal and plant | Low-affinity zinc ion transmembrane transporter activity | 15.679 | 1E-30 |
| Solyc03g096250.2 | Yippee zinc-binding-like protein (AHRD V1 **** C1FDK9_9CHLO); contains Interpro domain(s) IPR004910 Yippee-like protein | Zinc ion binding | -14.993 | 0E+00 |
| Solyc06g009240.2 | Cation/H(+) antiporter 15 (AHRD V1 **** CHX15_ARATH); contains Interpro domain(s) IPR006153 Cation/H+ exchanger | Sodium: hydrogen antiporter activity | -16.643 | 5E-30 |
| Solyc02g079710.2 | Serine/threonine kinase receptor (AHRD V1 **** Q7DMS5_BRANA); contains Interpro domain(s) IPR002290 Serine/threonine protein kinase | Transmembrane receptor protein kinase activity protein serine/threonine kinase activity | 16.627 | 5E-43 |
| Solyc02g080960.1 | TO62-3 (Fragment) (AHRD V1 ***- Q3LVL2_TAROF); contains Interpro domain(s) IPR010471 Protein of unknown function DUF1068 | | 16.565 | 2E-54 |
| Solyc02g079720.1 | Centrin (AHRD V1 **-- Q179F4_AEDAE); contains Interpro domain(s) IPR011992 EF-Hand type | G-protein beta/gamma-subunit complex binding | 16.190 | 5E-10 |
| Solyc01g017840.1 | subunit of replication protein A (AHRD V1 *-*- A6BLP0_IPONI); contains Interpro domain(s) IPR016027 Nucleic acid-binding, OB-fold-like | | -15.952 | 3E-79 |
| Solyc00g042640.1 | Helicase-like protein (AHRD V1 *-*- Q9AYF0_ORYSJ); contains Interpro domain(s) IPR010285 Protein of unknown function DUF889, eukaryote | | -15.877 | 3E-166 |
| Solyc04g064770.1 | Zinc finger CCCH domain-containing protein 38 (AHRD V1 *-*- C3H38_ARATH); contains Interpro domain(s) IPR000571 Zinc finger, CCCH-type | Zinc ion binding | -15.752 | 0E+00 |
| Solyc10g012020.1 | Unknown Protein (AHRD V1) | | -16.652 | 9E-33 |

| | | | | |
|---|---|---|---|---|
| Solyc03g096250.2 | Yippee zinc-binding-like protein (AHRD V1 **** C1FDK9_9CHLO); contains Interpro domain(s) IPR004910 Yippee-like protein | Zinc ion binding | -14.993 | 0E+00 |

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